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THE TYPES MITIS, INTERMEDIUS AND GRAVIS OF CORYNEBACTERIUM DIPHTHERIAE

A REVIEW OF OBSERVATIONS DURING THE PAST TEN YEARS[1]

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During the last fourteen years repeated references to peculiarly severe outbreaks of diphtheria have appeared in the medical journals and in the public health reports for various districts of Europe. One of the earliest of these was from Berlin, where Deicher (37) and Deicher and Agulnik (38) were amazed and chagrined to observe the diphtheria case death rate in the Virchow Krankenhaus rise from 5 per cent in 1924 to 26.7 per cent in the first five months of 1927. Although so high a case death rate has been uncommon, similar observations have been recorded in many quarters. This has been so in a quite special degree in Germany where it is emphasized amongst others by Königsberger (94), Wolter (198), Gundel (64), Paschlau and Sudhues (134), Degkwitz (36), and Woldrich (197). Rostoski (153) points out that in Germany the incidence of diphtheria rose from 30,000 in 1926 to 150,000 in 1936. Goeters (54) states that toxic diphtheria appeared in 1927 in a form which had not been observed for many years and has persisted ever since, while Spörl (168) notes that in Nürnberg there was a higher incidence of diphtheria in 1934 than in any year of this century. Outside Germany, however, there are also records of severe or peculiarly severe diphtheria in Czechoslovakia (45), in the north of France (108), in Italy (19), in Yugo-Slavia (166), in Hungary (198), in the British Isles at Leeds (2, 3), at Hull (98), at Cork (154), in the Ukraine (211), and quite recently in Dundee (178).

Not only are there these numerous records of unusually severe diphtheria, but there is no doubt that in many of these outbreaks the results of serum treatment have been singularly disappointing notwithstanding the great advances in the potency of antitoxic serum. So much is this the case that a considerable

controversy has gone on in the European medical press on the value of anti-diphtheritic serum. Thus Friedberger (49), Zischinsky (213) and Hottinger (82) have expressed marked skepticism about its value in the most severe types of diphtheria, and Paschlau and Sudhues (134), who report a 25 per cent mortality amongst malignant cases treated on the first day of disease, even suggest that in such cases serum is valueless unless administered before the second day. Many observers, however, have rallied to the defence of serum treatment, pointing out that the effect of serum is largely determined by the stage of the disease at which it is administered (6, 153, 188). The evidence for the value of serum and especially of its early administration can scarcely be questioned.

The skeptics have not, however, been easily silenced and reference has been made to Bingel's earlier work (1918) based on a series of over 900 cases in four s in which normal horse serum was found equal to specific antiserum in ment. Lastly, it is suggested that the argument about superiority of serum g proved by contrast of results from early and late administration is not tirely conclusive, since higher death rates appear in cases admitted late quite dependently of any serum therapy (70). On the other hand there are records f very large series of treated cases with extremely low deaths as e.g. Hecksher (74), who cites 4819 cases in Copenhagen with only 1.2 per cent of deaths after excluding 11 cases admitted moribund; and Widowitz (193), who records 1112 cases without a death. Von Bormann (14) brings evidence from two different lines of experimental work to dispose of the criticism that normal horse serum is equal to antitoxic sera in treatment of diphtheria. The first is that when these are tested in parallel experiment on diphtheritic infections of the guinea-pig's conjunctiva, it is found that there are three grades of infection varying with the dose administered: mild, moderate and severe. No distinction can be drawn between normal horse serum and antitoxin in the treatment of the first and last but quite a marked one in the group of animals receiving a moderately heavy injection. This difference is shown both in preservation of life and in healing of the eye intact. Not only is this the case, but if the antitoxin contents of various "normal" horse sera are titrated as well as their protective activities in curative experiments a direct ratio of antitoxin content to protective value is established.

There are, therefore, on the one hand observers of competence expressing skepticism about the value of standard diphtheria antitoxin in severe toxic diphtheria, founded on series of cases which are large enough to be significant, and on the other many series of cases in which the value of serum is clearly established, particularly when given early. What does this all amount to? There seems to be an unwillingness in some quarters to accept what is obviously the most probable explanation, i.e. that diphtheria in one place is different from what it is in another and that in the same place it may change over a period of years; and that however clearly established it is that over all cases serum is highly valuable, there may still exist a variety of diphtheria in which its effect is at best disappointing. Thus we have Leete, et al. (98) in England, Zischinsky (213) in Austria, Schiff and Werber (155) and Paschlau (132) in Germany and Anderson, Goldsworthy and Ward (4) in Australia all describing cases which

have died notwithstanding large doses of serum on the first day of disease. *This must mean that with certain varieties of diphtheria in certain patients it will always be impossible to give serum in time to be of use—that is so long as we are limited to the forms of antiserum now available.*

There are two other aspects of diphtheria as it has appeared in Europe in the last ten years, in which a change from the diphtheria general at the beginning of the century has been noted by most observers. These are a shift of the main incidence to school children from those of the age group 1 to 5 years, and a marked diminution of laryngeal diphtheria.

Age incidence. The evidence of the shift of the incidence from the infant group to that of the school children comes from all quarters in Germany and is very consistent. Thus in Hamburg, Bamberger and Lachtrop (7) observe a change about 1927. Before then there are two or three cases amongst very young children for every one in children of school age; but after 1927 the majority of cases occur in children of six years and above. Behr (9) at Plauen i/V observes the same rather later, 1933. In Lübeck the same shift occurs from 1927 onwards (54). Rostoski (1938) in a wide survey refers to increased incidence in the 6–14 years age group. In the Ruhr the incidence in children over 6 is three times as great as in those below it in the period 1934–36 (183). Spörl (1936) in Nuremberg observes that diphtheria mortality in the 1900–09 period was negligible above 9 years, whereas in 1933–35 it was maximal in the 8–9 years group and considerable in older children. Zischinsky (212) in a very extensive survey of diphtheria in Vienna from 1903–32 shows that in 1922, a year representing the lowest point in the curve of diphtheria incidence, the disease was still mainly in the 0–5 years group as in 1903, but that from 1926 onwards the main incidence moved to the older children. Similar observations have been made in Great Britain (35, 203); and in a recent comprehensive survey of diphtheria in Liverpool, Wright (204) records that in the period 1937–40 more than two-thirds of the diphtheria has been in children over 4 years of age. Cheeseman, *et al.* (20) have made an extensive statistical investigation of the possible causes of this shift of age incidence and are inclined to explain it on the grounds of decreasing congestion of the population dependent on the falling birth rate and general amelioration of social conditions which they argue lead to lessened exposure to contact with infection in earlier childhood and hence the arrival of a larger proportion of susceptible children in the schools. In support of this theory they point out that the high incidence of diphtheria in the younger age group was especially a phenomenon observed amongst the poorer classes in the community. This explanation, however, does not take into account the rather sharp coincidence between the appearance of the more toxic forms of diphtheria and the rise in age incidence which is a feature of so many of the reports from central Europe. It may well be, of course, that two or more separate factors have been responsible for this result.

Diminishing incidence of laryngeal diphtheria. It is obvious that since cases of obstructive diphtheria have always been more frequent in infants, any advance in the usual age for contracting diphtheria will automatically reduce the

number of laryngeal obstructions. When, therefore, there is talk of a decrease of laryngeal diphtheria which has coincided with an advance in the normal age incidence of the disease, it is important to determine whether the decrease in laryngeal diphtheria exceeds that which would naturally follow from change in age incidence.

Bamberger and Lachtrop (7), who devote a paper specially to the decrease in laryngeal diphtheria, bring forward figures which leave no doubt on this subject. In the 0-6 years group with a decrease of about one-third in total cases of diphtheria there is a five- to six-fold decrease in laryngeal involvement between the periods 1927–36 and 1917–26. In the older group, above 6 years, this is still more marked since with a two-fold increase of total diphtheria incidence, laryngeal diphtheria becomes about half as frequent as before. These writers also record that the decrease is not so much in the primary laryngeal as in the combined laryngeal and pharyngeal diphtheria.

Similarly Spörl (168) observes in Nuremberg a fall in laryngeal diphtheria from a maximum of 85% of all diphtheria in 1907 to less than 5% in 1934. It is altogether unlikely that shift of diphtheria from one age group to another was adequate to account for this. schinsky (212) in Vienna records 37% of croup complicating diphtheria in 1903, 28% in 922 and 13.2% in the period 1926–32 in the 0–4 years group of cases. It is therefore sufficiently clear that laryngeal diphtheria has decreased independently of the change in age incidence of diphtheria.

In contrast to all this, it is undoubted that in Denmark there had been a steady decline in mortality from diphtheria for many years up till 1931 (5). The contrast with North America is still more striking, for not only has diphtheria been nearly obliterated in big cities like New York, Toronto and Montreal, (102, 46, 47, 159) where comprehensive campaigns in prophylactic inoculation have been carried through successfully, but there has been a marked fall in the incidence of diphtheria throughout the country. Figures given in the *Journal* of the American Medical Association, 1938 p. 524, show that over the whole country there has been a drop in diphtheria mortality per 100,000 of population from figures which varied from 7 to 38 twenty-five years ago, to figures ranging from 0 to 6 in 1937 and that for a group of 88 cities with a total population approximating to 40,000,000 the rate had fallen from 10.84 in 1924 to 1.46 in 1937. Schumann and Doull (157) in discussing the phenomenon of decreased diphtheria mortality in Cleveland in particular and in other large cities of America recognize of course the part played by protective inoculation but doubt if that in itself adequately explains the phenomenon. They quote Frost's summary of this matter in the following terms: "One of the three changes tending to reduce morbidity, the decreased portion of Schick positives, may reasonably be attributed to artificial immunization. The other two more important factors, i.e. diminished infection frequency and the smaller ratio of cases to infections are not related in any obvious way to artificial immunity".

It is abundantly clear from consideration of the foregoing records that in the last twenty years there has sprung up a marked contrast between the incidence and severity of diphtheria in North America on the one hand and in many parts of Europe, and especially central Europe, on the other. What is the explanation of this contrast? Does it depend entirely on the more enthusiastic adoption of prophylactic inoculation in the New World? How far do questions of nutrition play a part? Are there complex and insufficiently understood aspects of the development of mass immunity independent of artificial prophylaxis which come into play or is it determined by weather periods appearing in the shorter 35-year cycles of cold moist weather and the longer 200-year cycles as suggested by Wolter (198)? Lastly may different varieties of the diphtheria bacillus endued with different epidemic potentialities be involved?

It was suggested ten years ago by Anderson, Happold, McLeod and Thompson (3) that there was ground for accepting variations in the nature of the diphtheria bacillus as a significant factor in the development of severe diphtheria in epidemic form. On the basis of a limited local experience it was shown that two distinct varieties of the *Corynebacterium diphtheriae* might be distinguished: *C. diphtheriae* type *gravis* and *C. diphtheriae* type *mitis*, the former more prone to produce toxic or malignant diphtheria and liable to epidemic spread, the latter associated more usually with mild and sporadic cases and dangerous to life chiefly on account of obstructive phenomena. Observations in a wider field soon made it clear that there was a third cultural group of diphtheria bacilli sharply defined from the others, often associated with severe toxic cases of diphtheria but less liable to epidemic spread than the *gravis* type and for this variety which has been very specially studied by Mair (104) and to which attention was drawn by him at the outset of these studies the designation *"intermedius"* has been suggested. In the last eight years this suggested classification has been a subject of study and controversy in all parts of the world and many new media have been introduced to facilitate the differentiation of these types (28, 69, 119, 170, 171, 176).

It is now possible therefore to make a definite statement about what is generally accepted with regard to the existence, nature and distribution of these types. There is, however, a good deal of diversity of opinion about their significance, and it will be necessary therefore to state the basis for such conflict of opinion as there is on the subject.

EXISTENCE, NATURE AND DISTRIBUTION OF THE GRAVIS, INTERMEDIUS AND MITIS TYPES OF THE DIPHTHERIA BACILLUS

The existence of these types has been so widely recognized and accepted that it cannot be considered to be any longer in doubt. It has been suggested from time to time, however, (a) that forms diverging from the three chief types described are sufficiently numerous and important in clinical diphtheria to make a wider classification desirable (18, 23, 24, 50, 51, 105, 106, 196, 202, 206).

On the other hand there are many areas in which apart from carriers and a few cases mostly mild all diphtheria has been attributed to one or other of the three types originally described in Leeds (1). Such observations come from northern Germany: Henneberg and Pels Leusden (75) who met only three out of 544 strains which were difficult to classify, and Grossmann (62) who met only five such strains out of 594 (also 26, 64); from Poland (209); from Khartoum (81); from Australia (4, 57, 58); and in England, from Hull (98), from Manchester (150), from Dundee (115), from London (104), and from Liverpool (204), where the most extensive of all recorded investigations of this kind has been carried out. There are a considerable number of observers who while accepting the existence of these types prefer to have them indicated by letters or numbers, as Wright and Christison (202) in England and Hammerschmidt (72) in Germany, who had already (in 1924) described types of the diphtheria bacillus corresponding to *mitis*, *gravis* and *intermedius* in some respects but had not suggested any differences in their pathological significance. This aspect of the question will,

however, be more appropriately discussed under the section on the significance of the types.

CRITERIA BY WHICH THE TYPES SHOULD BE DISTINGUISHED

In a general way, it may be said that where diphtheria has been mild and sporadic observers have found most difficulty in classifying the bacteria which they have handled in the three types *gravis*, *intermedius* and *mitis*, whereas in areas where diphtheria has been severe and epidemic the distinctions have been found easy. Thus in Great Britain atypical strains have been described notably in Stafford (106), in Edinburgh (202), in Glasgow (18) and in Newcastle (173), in all of which areas diphtheria was at the time either mild or only moderately active, whereas in many parts of central Europe where diphtheria has been for a number of years now of considerable severity very little mention is made of atypical strains.

The amount of work which has been done in the U. S. A. on this subject is very limited, the chief contributions being from Maryland (51, 137, 191, 192), from Illinois (63) and from New York City (159). In no case is the *gravis* type described as occurring in even 20 per cent of the cases and insofar as they go the reports suggest that *gravis* diphtheria as it has occurred in Europe has not yet been observed in the U. S. A. The descriptions of the *gravis* strains in Maryland and in New York City, most of which present only some of the characters described for *gravis* strains, suggest that they resemble those observed by the writer over a period of two years amongst material obtained from the Ruchill Fever Hospital in Glasgow. No single typical *gravis* strain was isolated in all this period, although a small percentage of starch-fermenting strains were found which varied in their cultural appearances on solid medium and broth from that of typical *mitis* strains to rougher cultures approximating more nearly to *gravis*. The serious diphtheria in this hospital throughout this period was associated predominantly with the *intermedius* type.

It is fairly obvious also that some observers are recording many more atypical strains than others because they have drawn a large proportion of the strains which they have examined from carriers rather than from cases of the disease, and also because they have insisted on too finely drawn distinctions. It is therefore desirable to define at this stage the criteria which have been found to be most reliable in differentiating these types. These are presented in table 1 in which the characters of primary importance for a rapid differentiation are italicized.

The statements in table 1 under 1, 2, 3, 4, 5 and 7 are based on observations made on many hundred strains gathered in England from Leeds, Hull, Manchester, Liverpool, Newcastle and London; in Scotland from Dundee, Edinburgh and Glasgow; in Wales from Cardiff; in Ireland from Cork; in Germany from Berlin and in Poland from Warsaw (1, 2, 3, 35, and unpublished work.)

The statement under 6 in respect of differentiation between *mitis* and *intermedius* is founded on the unanimous findings of many observers (22, 69, 144, 149, 195, 207). This is so generally recognised that Pesch (138) claimed the development of hemolytic activity in *intermedius* strains subjected to repeated subculture in broth as evidence of their mutation to *mitis*.

The statement under 8 in Table 1 is based upon figures cited in a later section of this review. These observations of the writer and his colleagues were made on a collection of over 300 strains gathered from a wide variety of sources in Great Britain, Ireland and Europe. The existence of considerable numbers of non-pathogenic *mitis* strains is generally admitted (22, 55, 56, 58, 59, 89, 104). The almost invariable pathogenicity of *gravis* strains is also widely acknowledged.

With regard to *intermedius*, the recorded observations are less consistent. In Mair's observations (104) they are the most constantly pathogenic of all types, whereas Wright

TABLE 1

Most reliable criteria in differentiating the three types of C. diphtheriae

	MITIS	INTERMEDIUS	GRAVIS
1. Morphology	*Long forms; metachromatic granules*	*Barred forms often long and clubbed at ends*	Short forms tending to stain uniformly and sometimes closely resembling Hofmann's bacillus
2. Appearance of growth on heated blood agar	Fairly abundant moist, relatively smooth, semi-opaque & glistening colonies	*Flat, fine dry, opaque, and associated with delicate olive green discoloration of medium*	Abundant, flat, dry, matted, relatively opaque
3. Appearance of growth on special blood tellurite media	*Smooth, convex, medium-sized, with black centre and semi-translucent grey periphery for first 36 hours. Finer and larger colonies*	*Flat, fine, dull with black centre and often small central papilla. Grey periphery with slightly raised margin. Colonies very uniform in size*	*Medium to large with slight to marked radial striations and slightly to markedly indented periphery. Color varying from grey-black to black. Finer and larger colonies*
4. Consistence of colonies	*Approximately that of warm butter, colony smears under needle and forms homogeneous suspensions*	Intermediate between *gravis* and *mitis*	*Approximate to that of cold margarine, colony is pushed in front of needle and tends to fracture*
5. Appearance of growth in nutrient broth	*Heavy uniform or mixed uniform and granular turbidity. Pellicle late, soft and leaving ring on side of tube*	*Finely granular turbidity, settling to leave clear supernatant*	*All variations from clear fluid with marked pellicle broken by agitation to coarse flakes which settle to base of tube to slight pellicle over abundant fine turbidity mixed with granules and flakes of varying size*
6. Hemolytic activity on blood agar plates	*Distinct*	*Absent*	Variable
7. Fermentation of starch and glycogen	*Negative*	*Negative*	*Positive*
8. Regularity of pathogenic action in guinea-pigs	10%-20% of non-pathogenic strains (high pathogenicity for mice)	10% non-pathogenic (low pathogenicity for mice and for spermophils)	Non-pathogenic strains extremely rare (moderate pathogenicity for mice)
9. Antigenic homogeneity or diversity	Great diversity of antigenic groups	Antigenically homogeneous	Two main antigenic groups each of which has been found as an epidemic strain over wide areas

(quoted by Stallybrass, 1936) finds about 30% and, Gregory (58) 64% non-virulent, while Rosa (152) observed about 30% of non-virulent *intermedius* amongst strains from carriers. It may be that peculiarities in these strains make the exact technique adopted more important in testing them, and Mair recognised this by using five times heavier suspensions of *intermedius* than of other types in his tests. It is interesting in this connection that they should have been shown to be the least pathogenic of the three types when tested on the spermophil (39) or on white mice (210).

The variations in the reactions of broth produced by the different type strains which were mentioned amongst the differential points in the first communication published on this

subject (3) are rather much influenced by variations in the composition of the medium and of conditions of incubation unless these conditions are very carefully standardized. They are important, however, since the marked tendency to alkalinization of *gravis* cultures may both mask fermentation and interfere with toxin formation.

The statements under 9 in Table 1 are based on the observations of Orr Ewing (49), Murray (117), Mair (104) and especially on those of Robinson and Peeney (151), who have made an extremely comprehensive survey of the immunological types of *gravis* strains.

Atypical Strains. If the suggested subdivision of the diphtheria bacillus into three main groups is accepted, then any strains which present a combination of the characteristics considered important in more than one group will appear as atypical strains. The chief variants from the well-defined types which have been described are the following:

(a) A strain resembling *gravis* in the colony which it produces on differential media such as that of Anderson, *et al.* (3), producing a marked pellicle in broth but failing to ferment starch and glycogen. Such strains have been described under the designation Type IV by Wright and Christison (202).

(b) A very smooth strain producing uniform turbidity in broth but fermenting starch; such strains formed a very small percentage in a series examined by the writer in Glasgow. Carter (18) also found them in Glasgow to the extent of 0.25% in 1600 strains; but 13% of such strains were observed (206) in a small series collected in South Africa, and one or two strains of this kind were found in New York (159).

(c) A strain resembling *intermedius* in colony and morphology but fermenting starch which has been described by Stuart (173).

Types V and VI of Wright and Christison (202) do not seem to require special consideration. They are only differentiated from the others by animal pathogenicity and there would be just as good reason to make non-pathogenic strains otherwise corresponding to *mitis* into Type VII, and non-pathogenic *intermedius* into Type VIII.

Atypical strains are only significant if they appear with sufficient frequency and are found to be definitely associated with serious clinical diphtheria. Judged by these criteria the only one of these forms which requires careful consideration is the rough strain which fails to ferment starch,—Type IV of Wright and Christison.

This variety of the diphtheria bacillus has been found in two of a small group of strains from Cyprus (202); in Glasgow (18) where it was responsible for less than 1% of cases in a large series; to the extent of 4% to 25% in diphtheria in Edinburgh (23, 24); in South Africa where they accounted for 22% of cases (206); in Victoria, Australia (59); in Liège, Belgium, where it was found in 13% of a small series of cases (189); in Leeds by the writer, where it has turned up at long intervals in a group of contacts and in the cases associated with them; and in New York City, where it appears to be not uncommon (159).

Atypical strains have been found in severe cases as well as more frequently in carriers in Edinburgh (23, 24). That they may play a part in significant clinical diphtheria is also evident from the work of Carter (18) who describes two moderately severe cases in Glasgow; and of Block (12) who later described in the same city a severe but limited epidemic apparently due to the consumption of ice cream infected by a carrier in which there were six deaths. From South Africa there were reported (206) three severe cases, one of them fatal, due to such strains.

It is quite clear, therefore, that on the one hand they have been found to be associated with severe and fatal diphtherias and on the other that they are

more common amongst carriers, convalescents and in diphtheria cases of more doubtful significance. Strains of this type—*gravis*-like colony and growth in broth but no fermentation of starch—provide a much larger percentage of non-pathogenic strains than do any of the three main types already described. What then is to be made of them? Are they to be described as a fourth distinct type or are they to be brought within the three main types already described? There are three probable explanations of these strains:

(a) That they are a distinct type as suggested by Wright, *et al.* If so it is one which has as a characteristic a very wide range of pathogenicity. (It is assumed that the only logical classification on the lines of Wright and Christison would be to combine their Types IV and VI as a single type.)

(b) That under this description two forms are being described, one of which is an atypical *gravis* strain which has lost its fermentative activity or developed it only feebly, and the second a form of little or no pathogenic importance. In favor of such a view are the observations of Robinson and Peeney (151) that one or two of the strains sent to them from Germany as *gravis* had lost the power of hydrolyzing starch but were identical in respect of response to agglutinating sera to the main European group of *gravis* strains; also the observation of Keogh, *et al.* (88) that strains of this type met in Victoria resembled the predominant *gravis* strain in Australia in their sensitivity to bacteriophages; and lastly that in Edinburgh the only city in which these diphtheria types have been followed from a period in which *gravis* was practically absent to one in which it was predominant a considerable increase in infections of this type immediately preceded the rapid advance of *gravis* infections (24, 201).

(c) That in each of the three main types there is a rough → smooth variation and that the rough are the more formidable variants in each group. In that case, Type IV of Wright and Christison would represent the extreme rough variation of the *mitis* strain. This theory is approximately that advanced by Clauberg, *et al.* (31). There is much to be said for this explanation, but it does not meet satisfactorily the existence of the large numbers of apathogenic strains of this kind. Extended work is necessary to elucidate the position of such strains.

Stability of the types in culture. The stability of bacterial types may be considered from two sides. It may be studied as a purely bacteriological problem or from the standpoint of the significance of the bacteria concerned in producing disease. Although these are two separate problems they are so far related that if the instability of these bacterial types in culture is shown to be extreme it is a waste of time to consider their significance in connection with disease. If on the other hand their significance in connection with disease is clearly established there may be ground for supposing that the demonstration of mutations under highly artificial conditions of culture is receiving a consideration and emphasis which is not justified. The question of the significance of the different types of the diphtheria bacillus has been recently discussed at great length by Morton (111, 112) and the general conclusion reached that (a) the *gravis* form of the diphtheria bacillus represents the smooth → rough variant in a sequence of changes that can be obtained with the diphtheria bacillus in the same way as they have been

with many other bacterial forms, (b) that a correlation between colony form and polysaccharide fermentation cannot be maintained. The last conclusion is based on the existence of a number of exceptions, and on the fact that Knox and Passmore (93) have demonstrated a limited absorption of oxygen in respiratory experiments in which suspensions of *mitis* strains are prepared in Ringer phosphate with 0.2 to 0.5 per cent of glycogen. The question of correlation of colony form and fermentative activity is clearly one of some importance since it is the point at which most attempts to produce mutations from *mitis* or *interdius* strains to *gravis* strains have broken down. Thus Christison (21), rter (18), Cooper, *et al.* (35) and Grossmann (62) have all failed to observe is mutation in collections of cultures kept in some instances for many years. eydel (162) in a large collection of cultures re-examined after a year in sublture observed one change of *mitis* to *gravis*, which she attributes to a culture itially mixed. It is well that the last possibility should be carefully considered n all rare observations of this kind occurring in large series of strains isolated from mixed bacterial cultures. Gins and Fortner (52) discussing the possibility of variation in diphtheria bacilli emphasize the importance of being on guard against prolonged symbosis of closely related bacterial forms and quote an instance in which specimens of paratyphoid B and *Spirillum alkaligenum* growing in mixed colonies could not be separated even after thirty subcultures from single colonies. Robinson (148), who has devoted much work to this subject, found that one out of 197 *gravis* strains had lost its capacity of fermenting starch on re-examination after 4 to 16 months in subculture, and did not observe any instance of acquisition of the power to attack starch among 395 non-starch-fermenting strains (*mitis* 120, and *intermedius* 275) in the same circumstances. He describes, however, one instance in which the colonies of an *intermedius* strain developed papillae, the subcultures from which after some months of training in starch-containing media gave rise to a starch-fermenting strain. Menton, *et al.* (106) and Leete, *et al.* (98) have also described a single strain in a large series which was atypical and had given rise to both polysaccharide-fermenting and non-fermenting subcultures.

Murray (118) described the transient acquisition of starch-splitting power by *mitis* and *intermedius* strains if these were grown in a rabbit-serum broth. This capacity never survived the first subculture and was obviously related to something carried over from the serum. Carter (18) failed to repeat this observation. Mair (104) is the only observer who has claimed to be able to develop this capacity for fermentation of starch easily in *mitis* and *intermedius* strains. His technique is to heat the cultures just short of the thermal death point and use the subcultures obtained from these. The writer made numerous attempts to repeat these observations but failed and has been unable to find any published corroboration of them.

This may all be summed up in the following sense: no artificial method of converting non-starch-fermenting diphtheria strains to starch-fermenting forms has been described that has been generally accepted. Where very large series of strains have been examined one or two have been detected which have shown

starch-fermenting capacity in a weak or irregular way. Lastly the education of a starch-fermenting strain from one originally non-fermenting has been achieved in one instance by methods similar to those by which in rare instances lactose-fermenting strains have been bred out from non-lactose-fermenting forms like the typhoid bacillus (181).

The observations of Knox and Passmore (93) on which Morton places special emphasis are maintained by him to show that there is no significant difference between the oxygen consumption per unit of dry weight of bacterial suspensions of the type *mitis* and *gravis* when employing glucose or glycogen as substrate! It is true that the amount of oxygen absorbed by the *gravis* strains in these experiments in the presence of glycogen was only some 50 per cent to 60 per cent greater than that absorbed by *mitis* strains in the same experiments, but since in a number of these experiments the controls for some strains showed as much or more oxygen absorption than did others in the presence of glycogen, their interpretation is at least open to doubt. This is the more so as no direct evidence was brought that glycogen was actually destroyed. It should not be overlooked that Passmore (135) in similar experiments got just as good evidence for the use of glucose and glycogen as substrates by *C. hofmannii*.

It would be about as sensible to argue from the latter observation that there was no correlation between glucose fermentation and pathogenicity in the group of corynebacteria as it is to state as Morton does on the basis of the former that there is no correlation between colony form and starch fermentation in the group *C. diphtheriae*. Hohn (80), Schlirf (156) and Warnecke (185) all agree in showing that *gravis* strains produce most acid in starch solutions and that *intermedius* strains produce much less acid in glucose than either of the other types.

Mutations other than those relating to fermentative activities. Many workers have investigated change of type as related to colony form, growth in fluid media etc., and the bacteria have been subjected to a variety of highly specialized conditions for this purpose such as growth in dilute solutions of $CuSO_4$ (142, 61), of LiCl (112), in various concentrations of different animal sera (92). In all of these ways it has been possible to get changes of colony form and of the character of the growth in broth, which are described as being of a more or less enduring character. The simplest of all methods of getting such changes appears to be repeated culture in fluid media at intervals of a week or more and carried on for a considerable period (21); and Pesch (138) claims that *intermedius* strains can be converted to *mitis* with regularity in this way, although he failed both by this and other methods to change *mitis* or *gravis* strains. These methods included culture in filtered sputum, recommended by Weigmann and Koehn (187) as a method by which conversion of type could be obtained in single-cell strains. As, however, these methods have led to conversions of diphtheria strains to *C. hofmannii*, a conversion which rather strains belief, and since the method of controlling the sterility of the sputum which is described is not very convincing, the conversions can only be accepted with some reserve. Lastly, there are quite a number of workers who either fail to observe any mutations at all in the course

of their observations of cultures or only record transient variations from which the bacterium readily returns to its original form as soon as it is allowed to grow in more favorable conditions (42, 61, 63, 75, 92, 144). Grossmann (62) who worked with single-cell cultures in sewage water failed to change *mitis* or *intermedius* but got some change of colony form in *gravis* without change in biochemical activities. Clauberg, *et al.* (31) are also among those who have gone ᵢ to this problem in a particularly thorough way using single-cell cultures, and ᵧ find that all altered strains return to type when transferred to a special ι-cystine medium. They maintain that observations such as those of ᵣhristison (21) and Hammerschmidt (72) only amount to rough → smooth ᵢᵣ ᵢⁿs within three distinct bacterial varieties.

One of the most extensive pieces of work on this theme has been the subject ᶠ a thesis in Holland (Siemens, 1938) in which the importance of examining well-isolated colonies is emphasized, a considerable number of atypical forms being brought into the three main groups when so investigated. The general conclusion is reached that *gravis*, *mitis* and *intermedius* represent distinct races and that the numerous variant and atypical forms described are pseudo-types *i.e.* forms of adaptation of the ground type to variations in external conditions. For Siemens, the differentiation of the *C. diphtheriae* to *gravis*, *mitis* and *intermedius* is as fully justified as that of the tubercle bacillus to human and bovine types. Although, therefore, opinion on the subject of the stability of the described types of *C. diphtheriae* is far from unanimous, it may perhaps be said that they have stood up as well to a very fierce flood of investigation as any other recognized bacterial groups. We are therefore examining races which are sufficiently stable and sharply defined in culture in the observations of most workers to enable us to form an opinion about their stability in the human and animal body. From the point of view of their significance in disease this is the most important aspect of the question.

ANTIGENIC GROUPS WITHIN THE TYPES

Work on this subject comes mainly from Ewing (44), Murray (117) and Robinson and Peeney (151). The execution of observations on agglutination of diphtheria bacilli is hampered because of difficulty in obtaining stable suspensions of *gravis* cultures; and a variety of expedients such as prolonged shaking (44) and the use of alkaline solutions instead of saline for preparing suspensions (117, 151) have been introduced. Most attention has been paid by these observers to the classification of *gravis* strains, but they all have recorded a number of observations on *mitis* and *intermedius*. There is good agreement that diversity of immunological groups is most marked among *mitis* strains. The findings about *intermedius* strains are more divergent, as Ewing in a small series finds 2 and Murray in a large one 4 serological groups besides 8 per cent of unclassified strains; whereas all strains of this type examined by Robinson and Peeney fell into one serological group; and the findings of Mair (104) are in accord. It may be that this difference is due to the fact that Murray based his findings entirely on agglutinin-absorption experiments. There is agreement that partial cross-agglutination between the starch-fermenting and non-starch-fermenting groups

may be observed, and Robinson and Peeney mention occasional *intermedius* strains which show considerable cross-agglutination with anti-*gravis* sera and even partially absorb these, an observation which was also made by Carter (18). Ewing, however, did not get cross-absorption, and also points out that the cross-agglutination is not reciprocal, the *gravis* strains failing to respond to *intermedius* serum. Murray found the three types quite distinct when agglutinin-absorption methods were employed.

All observers agreed in finding two or three principal serological groups of *gravis* strains and a few unclassified ones. Murray's groups comprise: (a) strains from Leeds, (b) from Hull and Cork and (c) from Glasgow, and Ewing's (a) strains from the London district and Leeds, (b) from Berlin and Britain, (c) from Hull and London, also 2 strains from Khartoum and 2 from Glasgow which do not fit into these. The work of Robinson and Peeney which is very comprehensive indicates the following groups; (a) strains from Britain and Australia, (b) from Cork, Europe and North America with rare strains in Britain, (c) predominant in Hull and found more rarely elsewhere in Britain, especially London, (d) from Scotland and Egypt, and (e) from Massachusetts only.

The only serious discrepancy in these observations is that Murray places the Cork and Hull strains in one group and Robinson and Peeney place them in different groups. This might be explained by a change of *gravis* strains in Hull, the two observers having obtained their samples at different times. It is to be noted that the Hull strains investigated by Leete, *et al.* (98) are culturally distinct from either the Cork or the Leeds strains. They give a finer, blacker and less rough colony when first plated on heated blood-agar-tellurite medium and they are less inclined to produce pellicle in broth, a finding which favors the observations of Ewing and of Robinson and Peeney rather than those of Murray. Actually Leete found that at a considerably later date (1939) the character of the Hull *gravis* strains had changed over to the typical *gravis* as first described in Leeds, and it was found by the writer that the strains then appearing agglutinated with an antiserum to Leeds *gravis*. This finding was confirmed by Robinson who determined those strains as belonging to his first group. The fourth and fifth groups of Robinson and Peeney do not correspond closely in cultural character to the original description of *gravis*, although they are starch-fermenters. Stuart's (173) unusual starch-fermenting strains from Newcastle were found by him to correspond to Ewing's group B, *i.e.* Robinson and Peeney's Central European group.

In toto these observations may be summed up in the sense that (a) the three types are serologically distinct, (b) each is made up of a number of serological sub-groups, (c) that the *intermedius* group is probably the most homogeneous, (d) that the organisms which fall strictly within the original description of *gravis* can be subdivided into two main serological subdivisions, one widespread in Central Europe and represented in North America, the other widespread in Britain (the recent outbreaks of *gravis* diphtheria in the east of Scotland are of this type), and that both of these have been found to be associated with severe epidemic diphtheria.

STABILITY IN THE ANIMAL AND HUMAN BODIES

The observations on stability in the animal body are in many respects the most reliable which are at our disposal since the bacteria are introduced into parts of the body where no other bacteria are normally present and there is therefore every probability that any bacteria recovered at a later date have been derived from those introduced earlier. Further the conditions approximate more nearly to those which the bacteria are likely to experience in the human body than

TABLE 2
Stability of the types in the guinea pig

OBSERVER	TYPE	NO. OF STRAINS	NO OF CULTURES RE-COVERED*	OBSERVATIONS ON CHANGE
Robinson (148)	*Gravis*	94	243	No significant change
Robinson (148)	*Mitis*	77	144	Two strains showed colonies and broth resembling *gravis*
Robinson (148)	*Intermedius*	111	287	Two strains showed irregularity in colony form, one resembling *gravis*. One strain already referred to is described in detail below†
Cooper, *et al.* (35)	*Gravis*	46	46	No significant change
	Mitis	26	26	No significant change
	Intermedius	13	13	No significant change
	Atypical	7	7	No significant change
Gundel and Erzin (65, 66)	*Gravis*	12	140	No significant change
	Mitis	13	92	No significant change
	Intermedius	7	84	No significant change
Zinnemann and Zinnemann (211)	*Gravis*	1‡		No significant change
	Intermedius	1‡		No significant change

* The number of cultures was greater than strains in some instances because more than one animal was injected with some strains and in many investigations cultures were recovered separately from various viscera.

† The behavior of this strain was very peculiar. It was the one already described yielding subcultures from papillae on its colonies which could be trained to ferment starch. Subcultures from the livers of guinea-pigs injected with this strain fermented starch; those from the local lesion did not unless specially trained.

‡ Passed consecutively through 30 guinea pigs.

those to which they are exposed in cultures. Observations on bacteria isolated at intervals from the human body, on the other hand, are almost always made from surfaces which are both the site of multiple infection and open at all times to implantation of fresh infections from without. The interpretation of changes of type of infecting bacilli on such surfaces unfortunately is a much more difficult matter since it is the question of stability in the human body which is the crux of the whole matter from the practical standpoint.

Observations in the animal body. Table 2 summarizes the observations made in the guinea pig.

Rabbit. Cooper, *et al.* (35) recovered 67 *gravis*, 24 *intermedius*, 47 *mitis* and 11 atypical strains after passage through the rabbit, but they showed no significant change.

Mouse. Pesch (138) passed 6 *gravis*, 6 *mitis* and 6 *intermedius* strains through mice without observing change. Murray (118) injected 16 *gravis*, 17 *mitis* and 16 *intermedius* strains, using 97 mice, and recovered a positive spleen culture from 44 animals. No change of type was observed. Zinnemann (210) passed 10 *gravis*, 16 *intermedius* and 10 *mitis* strains through mice and checked all strains recovered. No change of type was observed.

Rat. Seligmann and Jungeblut (160) were able to effect repeated passage of *mitis* and *gravis* strains in the rat by intracerebral injection without observing change of type.

General. Warnecke (185) also mentions stability of the types in animal passage; and Murray (116) and Siemens (165) resort to special methods, the former injecting strains into animals partially immunized to the same or different types, the latter introducing the bacteria in collodion sacs into the peritoneal cavities of guinea-pigs immunized against various types. Both of these procedures failed to elicit change of type. Nor were Anderson, *et al.* (1933) any more successful in obtaining change of type in the animal body when they resorted to the technique successfully employed by Griffith (60) with the pneumococcus.

Taken *in toto* these observations provide a substantial and remarkably consistent body of evidence in favor of the stability of these types in the animal body.

Observations in the Human Body. It is impossible or at least very difficult to get any conclusive evidence with regard to change of type by repeated examinations of the bacterial flora of the throat, nose, etc. for reasons already stated. There is ample evidence of the appearance of mixed infections and of the isolation of different bacillary types from the same patient at different periods of disease and convalescence. Are these changes to be attributed to cross-infection or to mutation? There is a wide variation in the extent to which change of type in the human body as observed in repeated cultures from the same patient has been recorded.

At Leeds, mixed infections were found (2, 35) to be extremely rare in acute cases of the disease, whereas in convalescents in the wards of fever hospitals they were not uncommon, infections with more than one type or change of infecting type being recognized in 40% to 50% of patients. In the Ukraine, 21% of such changes were observed in convalescents (211). Among 246 cases at Liverpool observed for a month or more in hospital 25% of instances of type change are recorded (53). Gundel (64) notes only a 1% change over all in a large series but a higher incidence of change among hospital cases; and Perry Whitley and Petran (137) in Maryland are exceptional in not observing any change of type on repeated examination. In Königsberg, change was observed in 21% of cases nursed in hospital and in 8% of those nursed at home (142). Kemkes and Steigler (87) found 32% in hospital cases and 10% in home nursed cases. In 19 cases examined in a Manchester hospital over periods up to three months, 11 showed change of type (148). The most extreme instances of type change are given by Hilgers and Thoenes (78), who recorded 85% at Magdeburg.

The fact that about three times as many changes of type have been observed in patients in hospital as in those nursed at home suggests that cross-infection may be a more important factor in such cases than change of type. This impression is borne out by many observations of diphtheria in limited or closed communities. Thus Mittag and Otto (110) describe an extensive although very mild infection of a group of scarlet fever wards in which 45 persons were involved and no change of type was observed over 6 months. Gundel (64) points out that out of 19 small villages in Brandenburg there were 17 in which only one type of the diphtheria bacillus was found. He also points out that where change of type occur in a hospital it is always in wards in which cases of both or all types have been introduced. Preuss (1936) describes an epidemic in a children's hospital of 23 cases all of which were due

to *mitis* strains; and the writer has observed a similar instance of an all-*mitis* epidemic among infants in a maternity hospital. Others (75, 122, 124) also agree that multiple cases in small institutions or in families are all of one type; and it is maintained (122) that where two types occur in one family it is usually due to attendances by children in different schools.

Several British observers have pursued the matter further, attempting to eliminate all possibilities of cross-infection and then observing the effect on the development of type change. "All *Gravis*" wards have been brought under observation in Manchester (148), and Leeds (35), and in these the bacteria disappeared more rapidly than in wards in which mixed cases, *i.e.* cases due to different types, were nursed; and no change of type was observed. Lastly, it was observed (205) over a period of ten months at Liverpool that among 63 cases (34 *gravis*, 12 *intermedius* and 17 *mitis*) nursed in separate cubicles no change of type was encountered. Emphasis is placed on the necessity of repeated examination of the nursing staff in such observations with a view to exclusion of carriers from the wards. Gundel (1936) in Germany also found that there was no type change in wards to which only *gravis* cases were admitted. A report (132) of two cases in which change of type was found although cross-infection could be excluded, was challenged (27) on the ground that the attendants on the patients did not have their throats and noses controlled bacteriologically; this was subsequently admitted (133).

It is apparent therefore that the available evidence is practically uniform in *ascribing the occurrence of type change in the human body to cross-infection.* Hence the presumption for stability of type in the human body is very strong.

To sum up: a very large number of observers have satisfied themselves about the existence of the three types and of the fact that they are usually readily and sharply defined and further there is much evidence for supposing them to be stable in the human and animal body. It is therefore profitable to go on to summarize the records of their distribution and their associations with clinical diphtheria in various parts of the world.

World Distribution of the Gravis, Mitis and Intermedius Diphtheria Types. There are only three areas in which the quality and amount of the work done is sufficient to give a fairly clear idea of the actual type distribution. These are Central Europe, Australia and Britain. In the remainder, the descriptions of the types published is insufficiently precise or the number of investigations published is too limited to allow of a clear definition of the type distribution.

The data on the geographical distribution of the three types of the diphtheria bacillus have been summarized in table 3.

This wide survey of the work on the types of diphtheria bacilli reveals one or two points of considerable interest. All over the world observers have for the most part found little difficulty in recognizing these types; but in areas where diphtheria is mild and infrequent there may be a rather large number of irregular or atypical forms as in Edinburgh (202); Stafford (106), Capetown (206), Baltimore (50), and New York City (159); whereas in areas visited by severe and epidemic diphtheria the bacterial types are easily and sharply defined: *e.g.*, Berlin (64), Kharkoff (211), and Leeds (3).

A further point of interest is, that with the exception of a short series in Warrington (107) and for the Posen area of Poland (209), no investigation has ever been recorded in which the *mitis* form has not been recognized. On the other hand, there are numerous areas in which the *intermedius* or the *gravis* form either

is not present or is so scantily represented that it escapes observation. Such are: the Sudan (81), South Australia (146), Amsterdam and Rotterdam (165), Posen district of Poland (209), Huddersfield (119), and Helsingfors (120), which

TABLE 3

Geographical distribution of the three types

COUNTRY OR AREA	NO. OF OBSERVATIONS	FINDINGS
Africa (Cape Town, Sudan)	Small	*Mitis* predominant in Cape Town and atypical forms unusually numerous. No *intermedius* in either area (81, 206)
Australia	502	*Gravis* slightly predominant all over; *intermedius* predominant in Melbourne and absent in Adelaide (4, 57, 146, 194)
Canada	Very small	All three types have been recognised (151, 202)
Cyprus	Very small	No *gravis* (202)
Denmark	Very small	No *gravis* (129, 151)
England, Wales	12,282	*Gravis* predominant in Yorkshire 1930–34 and later in Lancashire. *Intermedius* predominant in south and west. *Mitis* predominant in Staffordshire. Fairly even mixture of types in London area, in east Yorkshire after 1934 and in Northumberland (34, 35, 83, 98, 104, 106, 107, 119, 149, 150, 174, 200, 204)
Finland	Small	*Gravis* +; no *intermedius* (120)
France		No information (100, 127, 147)
Germany	4,696	All three strains found in all areas, but *gravis* markedly predominant in east and north-east and to a slight extent in other areas (8, 20, 22, 62, 64, 68, 75, 76, 78, 90, 122, 132, 141, 144, 155, 170, 185, 190)
Holland	521	*Gravis* predominant in Rotterdam, *mitis* in Amsterdam (165, 177)
India	Very small	No *gravis* detected (151)
Ireland (Dublin, Cork)	Small	*Gravis* markedly predominant (35, 40)
Italy	Small	All three types and atypical strains recorded (16, 48, 139, 152, 167, 184)
Poland	841	*Gravis* predominant; *intermedius* absent except in Lemberg (91, 161, 209, 215)
Russia	Small	*Mitis* predominant and *gravis* scanty in Moscow. *Gravis* predominant at Kharkov two years later (163, 211)
Scotland	2,626	*Intermedius* predominant 1934–39 then a swing over to *gravis*; atypical strains numerous in Edinburgh (18, 24, 35, 115, 178, 203)
Spain	Very small	*Gravis* present (25)
Switzerland	Small	All three types and atypical forms (196).
U. S. A.	Small	Typical *gravis* strains rare (50, 51, 63, 79, 137, 151, 159, 191, 202)

have been found to be free of *intermedius* infections; and Edinburgh (201), Copenhagen, New York City and India (151) in which over certain periods at all events *gravis* strains have not been found.

Another noteworthy feature is that there are at least ten of the areas in which investigations have been made that are found to be dominated with *gravis* infection to the extent of 70 per cent or more of all diphtheria occurring. The same has only been observed with *mitis* in Amsterdam (165), and with *intermedius* in Bristol (34), although Glasgow approached closely to 70 per cent of *intermedius* infection in 1932–35. It would seem, therefore, that whereas *mitis* diphtheria is generally endemic, *gravis* diphtheria and to a less extent *intermedius* diphtheria have an epidemic spread. It may be, of course, that what we are witnessing is a gradual spread of *gravis* infection over the world with manifestations of epidemic incidence wherever it appears for the first time.

RELATIONS OF TYPE OF C. DIPHTHERIAE TO CLINICAL SEVERITY OF CASES

The observations which had accumulated on this subject were reviewed by Cooper, *et al.* in 1936; and in a group of about 6,000 cases recorded mainly in Britain and Germany the relative death rates were found to be *gravis* 13.3 per cent, *intermedius* 8.6 per cent and *mitis* 2.3 per cent. Hemorrhagic phenomena appeared to be slightly more common in *intermedius* than in *gravis* infections, whereas paralytic phenomena were definitely more common in *gravis* infections. Paralytic phenomena were less frequent and hemorrhagic phenomena rare in *mitis* infections. The *mitis* strains were, however, responsible for about four times as many cases of laryngeal involvement as the other strains. A summary of the observations which have accumulated since that review are presented in table 4.

From this it appears that in so far as the groups of cases investigated are concerned much higher case death rates have been associated with *gravis* strains than with the others in Australia, Russia and Poland. The highest case death rates are also associated with *gravis* strains in Scotland, in Germany and in the U. S. A., whereas in England in the three districts examined during the period 1936–38 the case death rate from *intermedius* infections has been higher than that for *gravis*. The figure for *mitis* case death rate is for the collected observations much the same as before, falling between 2% and 3% but there are three instances in which an unusually high case death rate has been recorded. One of these is from Australia (4) recording an 8% case death rate. In this instance, however, there appears to have been selection of the more serious cases for detention in the hospital concerned. The other figures from Australia come into line with wider observations on the lethality of this type. The second is from Maryland, where Perry, Whitley and Petran (1936) record a *mitis* case death rate of 8.2%. Unfortunately there are no other records of this kind which would enable us to judge whether that was characteristic of American *mitis* infection or an exceptional happening. In the third instance, the observations (78) at Magdeburg of a case death rate of 19% in *mitis* infection are so completely out of line with all other observations made in Germany and elsewhere that some doubt is left about their validity. This is the more so when it is noted (a) that appearance of colony alone was the criterion for differentiating those strains and (b) that the number of changes of type during the patient's stay in hospital was greater than those recorded in any other series. Preuner (143) makes the interesting observation n connection with these findings that in *mitis* infections the case fatality rises proportionately to the amount of cross-infection in the series of cases. This is not observed with other types.

The second series is rather overweighted with the English observations which amount to more than half of the total, and it is an open question whether a truer picture of the relative severity of each strain would be got by considering all recorded results or by taking a repre-

TABLE 4

Observations on the correlation of clinical severity and type of C. diphtheriae infection recorded since earlier results were summarized by Cooper, et al. (1936)

AREA	REF.	GRAVIS			INTERMEDIUS			MITIS			ATYPICAL		
		Cases	Deaths	%	Cases	Deaths	%	Cases	Deaths	%	Cases	Deaths	%
Australia	(4)	96	25					88	7				
	(57)	12	1		25	1		12	0				
	(194)	109	5		13	1		47	1		31	0	
	(146)	32	5					36	2				
		249	36	14.5	38	2	5.2	183	10	5.5	31	0	0
Scotland													
Glasgow	(18)	93	8		1052	58		239	4		80	1	
Edinburgh	(201)	640	33		473	16		153	2		146	11	
									(type IV)				
		733	41	5.6	1525	74	4.8	392	6	1.5	226	12	5.3
England													
Essex	(174)	253	24		87	12		42	2		17	0	
Leeds	(200)	780	50		157	17		975	18				
	(33)	11	0		447	9		82	2				
Liverpool	(204)	4126	260		1518	162		2395	53				
Huddersfield	(119)	48	3		1	0		5	1				
		5218	337	6.5	2210	200	9.0	3499	76	2.2	17	0	0
Germany	(190)	71	5		37	2		6	0				
	(78)	53	1		17	0		78	15				
	(90)	42	3		82	7		26	0				
	(87)	279	9		124	1		131	0				
	(75)	60	3		41	2		13	0				
	(144)	10	1		19	0		36	0				
	(124)	13	0		16	1		38	2				
	(185)	18	1		7	0		29	2				
	(141)	779	19		139	1		47	0		35	0	
	(143)							200	5				
	(8)	52	9		19	3		14	1				
	(122)	86	3		128	9		155	5				
	(64)	692	54		102	6		37	0				
		2155	108	5.0	731	32	4.4	810	30	3.7	35	0	0
Poland	(91)	55	10					52	2				
	(209)	300	45										
		355	55	15.5	0	0	0	52	2	3.8			
South Africa	(206)	6	0					65	1		27	2	
		6	0	0				65	1	1.5	27	2	7.4

TABLE 4—*Continued*

AREA	REF.	GRAVIS			INTERMEDIUS			MITIS			ATYPICAL		
		Cases	Deaths	%	Cases	Deaths	%	Cases	Deaths	%	Cases	Deaths	%
Russia	(163)	6	1		28	2		64	6		3	1	
	(211)	183	24		2	0		66	1				
		189	25	13.2	30	2	6.7	130	7	5.5	3	1	
U. S. A.	(137)	14	2		101	2		134	11				
	(63)	2	0		48	1		1	0		1	0	
	(159)	3	1					17	1		11	0	
		19	3	12.5	149	3	2.0	152	12	8.1	12.0	0	
Holland	(177)	184	8		94	1		81	3				
	(165)	71	5		37	2		6	0				
		255	13	5.1	131	3	2.3	87	3	3.4			
Totals		11492	926	8.1	6807	487	7.2	6858	181	2.6	663	24	3.6
Per cent of type incidence			44.51			26.36			26.56			2.57	

sentative 500-strain sample from each of the larger collections and combining these with similar or approximately similar samples from each of the smaller collections. If the whole of the English results are incorporated, the *intermedius* infections appear to be more severe; if the latter course is followed the *gravis* strains are shown to be responsible for a case death rate twice as great as that of *intermedius*.

A combination of all available observations on the clinical significance of the types, *i.e.* this series together with those published in 1936 (35), gives a grand total of about 25,000 cases. Of these, there are 11,492 due to *gravis*, with 8.1 per cent case mortality, 6,858 due to *mitis*, with 2.6 per cent case mortality, and 6,807 due to *intermedius* with 7.2 per cent case mortality. As judged by case mortality, the *gravis* strains still lead although the *intermedius* strains are very near them, and they are three times as dangerous as the *mitis* strains.

Among observations accumulated since 1936, full clinical details of the cases are only available for those from Leeds and Liverpool which with the addition of a few made in Germany and Hull provide the results shown in table 5.

These results indicate that the *intermedius* type is most severe in respect of hemorrhagic, paralytic and cardiac complications. This is mainly due to the predominance of Liverpool results as *intermedius* infection in this city has been peculiarly severe. If a similar analysis of the cases from Bristol had been available and equal numbers of cases from that area had been included, a result much more like the earlier one would be obtained. This showed that the *intermedius* infection was most often associated with hemorrhagic phenomena but less responsible for paralytic manifestations than the *gravis* strains. This is also the

final result obtained when both series are combined as in table 6. It is interesting that the impression gathered in the earlier series that *intermedius* infection was more often associated with hemorrhagic phenomena is considerably emphasized by the second series.

The Morbid Anatomical Picture Associated with the Different Types of C. diphtheriae. Very little appears to have been done on this subject apart from a series of post-mortem examinations carried out by Clauberg and Plenge (32) and another by the writer with Orr and Woodcock (103). The latter series included 51 cases (11 *mitis*, 14 *intermedius*, 23 *gravis* and 3 mixed) and all macroscopic and microscopic examinations of the viscera were checked by bacteriological investigations in order to determine (a) that the type of bacillus originally isolated from the patient was the one present at death, (b) how far the bacilli had penetrated from the original focus in the upper respiratory tract, (c) whether

TABLE 5

Relation of types to hemorrhagic and paralytic phenomena, myocardial lesions and laryngeal involvement

| | PERCENTAGE ASSOCIATION WITH | | | |
	Hemorrhagic	Laryngeal	Paralytic	Cardiac
Gravis	1.2% of 1622	2.5% of 1784	13.1% of 1914	8.2% of 1914
Intermedius	2.1% of 728	2.3% of 859	15.4% of 879	12.4% of 879
Mitis	0.1% of 1758	6.9% of 1845	6.03% of 1890	2.5% of 1890

TABLE 6

| | PERCENTAGE ASSOCIATION WITH | | | |
	Hemorrhagic	Laryngeal	Paralytic	Cardiac
Gravis	2.2% of 3118	2.4% of 3066	14.9% of 3648	8.7% of 1988
Intermedius	3.2% of 2175	1.7% of 2090	11.9% of 2358	12.4% of 879
Mitis	0.2% of 2572	7.1% of 2534	5.5% of 2738	2.5% of 1890

secondary infection played any important part in the death of the patient. The examinations were made in Leeds more than two years after a sharp epidemic of *gravis* infection, and during this period there was a falling incidence of *gravis* infection and a rising incidence of *mitis* infection. Apart from a sharp epidemic localized to one school and responsible for six deaths, *intermedius* infections were scanty during this period and the material from such cases was supplemented by specimens obtained through the kindness of Professor H. D. Wright from deaths occurring in Liverpool. There were only three cases in which evidence of cross-infection was obtained. Lesions very similar to those described for *gravis* infections below are recorded by Kroemer (95) in a series of ten fatal cases of toxic diphtheria. The strains were typed in only four cases of this series: three were *gravis* and one was *intermedius*.

The only interesting observations with regard to the possible significance of secondary infection in the observations of McLeod, Orr & Woodcock was the invariable association

of *C. diphtheriae* and *Hemophilus influenzae* in all patchy hemorrhagic lesions of the lungs; these were found most often in *intermedius* infections. No evidence of distribution of *C. diphtheriae* through the tissues has been obtained except that the *gravis* strains have been recovered from areas of the lung most remote from the bronchi with more frequency than other types. Others, however, submit evidence, post-mortem or by blood culture, for the existence of bacteriemia in severe diphtheria (166, 32, 101, 86, 56). In these instances the type, when determined, has been *gravis*. The only *mitis* septicemia described is one noted by Heubach (77) in a mixed infection with streptococcus from which the patient recovered.

There was a definite contrast between the lesions of *gravis* and of *mitis* infections in the following respects: the tendency to damage of the underlying tissues in the area of the local lesion was much more pronounced in *gravis* infection. This was specially noteworthy in the tonsil in which necrosis and hemorrhage was the rule, and the process usually extended to the tissues of the tonsillar bed and to the cervical lymphatics. There was also a reasonably close correlation between the extent of these tonsillar lesions and the amount of focal necrosis present in the lymphoid tissue of the malpighian corpuscles of the spleen. Lesions of the suprarenals were not a feature in this series. In respect of the spleen lesions and absence of suprarenal lesions, Clauberg and Plenge's (32) series agrees. By contrast the deep tissues were relatively normal in *mitis* infections and the reaction consisted mainly in abundant production of fibrinous membrane which extended more often to the larynx and tended to invade the bronchial tree more frequently. These facts notwithstanding, there was little microscopic evidence of penetration of the deeper tissues by the *gravis* strains (cultural evidence was obtained in the lungs as already mentioned) and it would therefore appear that the distinction in lesions was due to a difference in quality or quantity of toxin produced. This conclusion is reached by Kurkhaus (96) who describes similar tonsillar lesions in cases of diphtheria which die early, although he does not go into the question of bacillary types.

These findings are of course in keeping with the universal experience that *mitis* strains are most often associated with obstructive lesions in the air passages. The lesions produced by the *intermedius* strains corresponded in type to those associated with the *gravis* strains but were less pronounced.

Preuner (143) refers to the special connection of severe and fatal cases of *intermedius* infection with bronchopneumonia but does not give any figures on which this suggestion is based. In our series of post-mortems, the bronchopneumonias were more common in the *intermedius* than in the other infections but the difference was not great enough to be significant. An extended series might bring it out more clearly.

There is occasional mention of *intermedius* deaths complicated by bronchopneumonia (63, 155). Wright (204), however, analyzing 475 deaths from diphtheria in Liverpool finds only 2 pneumonia deaths recorded in *intermedius* infections as compared with 8 out of 53 in *mitis* infections and one out of 260 in *gravis* infections. Naturally the *mitis* strains with their tendency to obstructive lesions of the respiratory tract and consequent operative interference are specially prone to pneumonic lesions and five bronchopneumonias are mentioned in the first 16 recorded *mitis* deaths (35). The ground for supposing that the *intermedius* strains are associated with bronchopneumonia independently of obstruction of the upper air passages to a greater extent than the other strains is not therefore definite although it may depend on some special bacterial association which was not active in Liverpool in the period of investigation.

Bamberger and Lachtrop (7) mention a fall in termination of diphtheria cases by bronchopneumonia from 28 in 1925 to none in 1936. Such a change in view of Wright's figures might easily be associated with a turn over from *mitis* to *gravis* diphtheria.

Two recent writers on the pathology of diphtheria (Watjen and Reimann, 1937) without going into the question of types differentiate (a) malignant toxic diphtheria with lesions such as we have described in *gravis* and to a less extent in *intermedius* diphtheria and (b) banal diphtheria in which early death is by obstruction and which corresponds in post-mortem appearances to infections of *mitis* diphtheria.

THE CARRIER PROBLEM IN THE LIGHT OF THE DISTRIBUTION OF BACTERIAL TYPES

The literature on this subject is difficult to analyze because many authors have made no distinction between nasal diphtheria and the carrier estate. Others do not differentiate between contact carriers and carriers in the community at large who have had no direct contact with cases of the disease. The latter distinction is important (26).

Without making these discriminations, however, and by simply gathering up all recorded observations on types of *C. diphtheriae* found in individuals who did not give a history of a recent attack of the disease we get the results recorded in table 7 in which incidences of types in cases on the one hand and in carriers on the other are compared.

In a number of instances only percentages of the different types of carriers are given but when all the actual figures recorded are summed up the result is: 388 or 31.4 per cent of *gravis* carriers, 513 or 47.5 per cent of *mitis* carriers, 146 or 13.5 per cent of *intermedius* carriers, 82 or 7.6 per cent of carriers of atypical strains.

By contrast the figures for the whole series of cases recorded were: *gravis* 45.5%, *mitis* 26.8%, *intermedius* 25.2% and atypical, 2.6%. These combined figures for type incidence, therefore, indicate that the *mitis* and atypical groups are found oftener in carriers than in cases, whereas the reverse holds for the *gravis* and especially for the *intermedius* groups. This is equally clear from observing the detail in table 7. Only 4 out of 17 observers find the *gravis* incidence in carriers distinctly higher than in cases; whereas in one instance only (22) in Berlin is a considerably higher incidence of *intermedius* in carriers than in cases recorded. With *mitis* infection the reverse holds, since 15 out of 17 observers find a higher incidence of that type in carriers than in cases.

Wright (204) is the only observer who has carefully examined the persistence of the various type infections in convalescents in a considerable series of cases in which cross-infections have been excluded. These observations indicate that *gravis* infections are the most persistent and that *intermedius* infections clear up most quickly, whereas *mitis* infections are a little less slow than *gravis* ones in disappearing. These observations would explain satisfactorily the relative scarcity of *intermedius* carriers and the lesser epidemic tendency of this type of diphtheria notwithstanding its severity. They do not however explain the relatively higher *mitis* carrier rate. Of course it may well be that the conditions in Liverpool, in which in fact only a slight excess of *mitis* carriers over cases exist, do not fairly represent the conditions which exist more generally.

Both Mair (104) in London and Hertel (76) in Leipzig describe the *mitis* carrier infection as the most persistent. Hertel attributes this to the greater tendency shown by this type to produce lesions of the antrum and other air sinuses of the head. Clauberg (26) has made the practical suggestion that, as far as isolation of carriers is concerned, we should concentrate on the *gravis* carrier and possibly also the *intermedius* carrier since these are rarer and more dangerous, leaving the more numerous and less dangerous *mitis* carriers alone. He bases this suggestion both on his own observations in Berlin and on that of Dudley, et al. (41) that the presence of *gravis* carriers in a closed and immunized community led to a mild outbreak of diphtheria, whereas the presence of *mitis* carriers both before and after this event left the school free from any clinical manifestations of illness. The observation of Otto and Mittag (123) that *mitis* infection ran through 45 patients in a scarlet fever ward without causing any clinical diphtheria is evidence in the same sense. Clauberg's suggestion has been considerably discussed in Germany, where he is supported by Preuner (141) and by Kleinschmidt (90) who records that in a small series of family contacts of diphtheria convalescents one case of diphtheria occurred for every 3 carriers which resulted from *gravis*

TABLE 7
Contrast of type incidence in cases and carriers

PLACE	GRAVIS	MITIS	INTERMED.	ATYPICAL	REF.
Stafford	17	50	0	33 % in cases	(106)
	18.5	55	0	26.5 % in carriers	
Manchester	51.7	28.1	20.2	% in cases	(150)
	30.5	53.4	16.1	% in carriers	
Liverpool	34	41.5	24.4	% in cases	(164)
	34.8	44.7	18.9	% in carriers	
Dundee	6	37	57	% in cases	(115)
	12.5	87.5		% in carriers	
Edinburgh	0	16	50	34 % in cases	(23)
	0	20.2	20.2	59 6 % in carriers	
Berlin	77	10	5	8 % in cases	(22)
	44	32	15	9 % in carriers	
	72	25	3	% in cases	(26)
	28	70	2	% in carriers	
Prussia	79	16	5	% in cases	(68)
	50	29	6	15 % in carriers	
	83	5	12	% in cases	(64)
			Gravis carriers stated to exceed *mitis* carriers		
Königsberg	82	4	14	% in cases	(141)
	64.3	10.2	13.2	% in carriers	(142)
Düsseldorf	15	55	29	% in cases	(144)
	27	53	20	% in carriers	
Leipzig	61	17	22	% in cases	(8)
	80	13.3	6.7	% in carriers	
Berlin	60	27	13	% in cases	(132)
	45	49	6	% in carriers	
Cologne	28	17	55	% in cases	(90)
	24	35	41	% in carriers	
	33	54	13	% in cases	(185)
	0	95.2	4.8	% in carriers	
Belgium	63	18	2	17 % in cases	(189)
	50	50		% in carriers	
Holland	50	22	25	3 % in cases	(177)
	14.3	74.3	8.6	2.9 % in carriers	
Russia	5	60	31	4 % in cases	(163)
	10	90		% in carriers	
Australia	24	24	50	% in cases	(57)
	3	61	36	% in carriers	(55)

contact, one case for every 2 carriers from *intermedius* contacts but only one case for every 12 carriers resulted from *mitis* contacts. In spite of this evidence from his own work favoring Clauberg's suggestion, Kleinschmidt goes over to the majority who conclude (13, 132) against this suggestion, because (a) *gravis* carriers are more numerous than Clauberg supposed, and (b) some workers have recorded a number of severe *mitis* infections and hence this type must be considered potentially dangerous. Clauberg (29) taking up some of these criticisms points out that his suggestion was not put forward as an ideal course but as the best expedient in view of the large number of carriers and the limited laboratory facilities available. The suggestion does not appear to have been discussed directly outside Germany. There is a good deal to be said for it if the *mitis* carrier concerned is not likely to make any considerable contact with children in the 0 to 4 age-group since among these alone does *mitis* cause any significant number of deaths (204).

RELATIVE CAPACITY OF THE DIFFERENT TYPES TO PRODUCE CLINICAL DIPHTHERIA
IN SHICK-NEGATIVE AND INOCULATED PERSONS

A considerable number of observations have been made on this subject in Britain but relatively few elsewhere (see table 8). Puckey (146) records a few typed cases of diphtheria in immunized persons in Australia. *Gravis* infections of the immunized are mentioned in Berlin (30), in a country district of Austria (97), in Cork by Saunders and the writer, and very recently by Tulloch in Dundee. In so far as they permit of numerical statement these observations are collected in table 8. This table includes the naturally Shick-negative, those who had become Shick-negative after immunization, and those immunized but not finally Shick-tested.

It is clear from table 8 that there has been no record of significant diphtheria in the immunized and in the Shick-negative apart from the *intermedius* and *gravis* strains; and apart from the experience of Liverpool, where the *intermedius* infections have been particularly severe, diphtheria in the immunized has been dominated by *gravis* infection. Only the accumulation of observations on a larger scale in a number of different centres will finally settle the relative importance of these two types in this respect.

It is, of course, true that one cannot expect to get diphtheria in the immunized from *mitis* strains in an area like Cork where they are rare; but it is also true that in such an area, *i.e.* one with an almost complete predominance of *gravis*, there has been one case in the immunized to every five in the non-immunized population, whereas in Stafford (107) with the highest proportion of *mitis* infection recorded in any large series of observations the cases in the immunized are in the ratio of 1 to 100 in the non-immunized.

RELATIVE PATHOGENICITY OF THE DIFFERENT TYPES IN THE ANIMAL BODY

The techniques followed by the different observers in this field have varied widely and it is not to be expected that their results should coincide exactly. For example Mair, who finds the *intermedius* strain more constantly pathogenic than any other type, always injected five times as much of these strains as he did of the others. In our own observations, on the other hand, in which *gravis* strains were found to give 100 per cent positive results, the combined results of subcutaneous injection in the guinea-pig and intramuscular injection in the rabbit are considered, and 1/4 to 1/8 of the suspension from an 18-hour Löffler

TABLE 8

Cases of typed diphtheria in the Schick-negative and in the immunized population

AREA	TYPE	PER CENT INCIDENCE OF THE TYPE OF DIPHTHERIA IN THE AREA AT THE TIME OF OBSERVATION	SEVERITY OF CASES					REF.
			Fatal	Severe	Moderate	Mild	Total	
Manchester	*Gravis*	52	3	6	7	10	26	(150)
	Intermed.	20			5	4	9	
	Mitis	28						
Stafford	*Gravis*	4						(107)
	Intermed.	20						
	Mitis	68					5*	
	Atypical	8						
Leeds 1931–36, (mostly 1934–36)	*Gravis*	77		3	21	41	65	(182) (35)
	Intermed.	10		3		8	11	
	Mitis	13			1	7	8	
Leeds 1938–40	*Gravis*	54		2	15	33	50	(200)
	Intermed.	7				6	6	
	Mitis	39		1	5	29	35	
London	*Gravis*	Not known				14	15†	(131)
	Intermed.	Not known						
	Mitis	Not known						
Liverpool	*Gravis*	36		8		46‡	54	(164)
	Intermed.	25	2	11		24‡	37	
	Mitis	38				17‡	17	
Australia	*Gravis*§	47	1	3		1	5	(146)
	Mitis§	53			1	2	3	
Cork	*Gravis*	91		2	5	9	16	(182)
	Intermed.	4						
	Mitis	4			1		1	
	Atypical	1						

* There is no statement about the severity of these cases.

† 1 not classed clinically.

‡ The figures in the Liverpool group bracketed as moderate or mild have been classified as mild in this summary.

§ Figures include diphtheria in patients with history of previous attacks.

Summary: In all there were 231 *gravis* infections of which 4 were fatal, 24 severe, 48 moderate and 154 mild; 63 *intermedius* infections of which 2 were fatal, 11 severe, 8 moderate and 42 mild; 68 *mitis* infections of which none were fatal, 1 was severe, 7 moderate and 55 mild.

Of the 362 cases of diphtheria occurring in Schick-negative and immunized patients 63.8% were *gravis*, 17.4% *intermedius* and 18.8% *mitis*.

slope culture was injected to each animal. A number of observers again restrict their records to the widely accepted method of the intracutaneous injection of small amounts of bacterial suspensions, as for example Parish (128). Others again (106) have used broth cultures.

It is relevant at this point to ask what exactly it is that is being determined in these tests. For Turewitsch and Kotschetowa (180) the intracutaneous virulence test is essentially a test of capacity to produce toxin *in vitro*. This is probably mainly true, but it seems likely that the results recorded by various workers indicate toxin produced *in vitro* in some cases, *in vivo* in others and most often a combination of both.

Results in guinea-pigs and in a few instances in rabbits; subcutaneous and intracutaneous injection. A general impression of the relative pathogenicity of the different types may be taken from the combined results obtained in Australia, China, England, Germany, Italy, Poland, Russia and the Ukraine (22, 35, 55, 56, 58, 65, 89, 104, 106, 128, 137, 149, 152, 169, 184, 185, 192, 194, 207, 211). Atypical strains: 97 examined, 53.6 per cent found virulent. *Gravis* strains: 2697 examined, 98.1 per cent found virulent. *Intermedius* strains: 2483 examined, 96.5 per cent found virulent. *Mitis* strains: 3634 examined, 84.6 per cent found virulent. Among these observations, series with 100 per cent positive results were obtained in Berlin, Bologna, Hamburg, Kharkoff, Leeds, Manchester, and Maryland with *gravis* strains; in Berlin, Hamburg and Maryland for *intermedius* strains, but are not recorded anywhere for *mitis* or atypical strains. It is noteworthy that the highest figures for virulence in the three main types appear in the series in which the largest numbers have been examined. Two factors which tend to explain this are probably (a) that large numbers of observations are more readily accumulated where an epidemic spread is taking place, and (b) that observers who have recorded large series of tests are more experienced both in typing and in virulence tests.

The general conclusion may safely be drawn from these very extensive observations that on *gravis* and *intermedius* strains which appear to *experienced observers* to be typical, virulence tests are superfluous.

Observations on unusual animals, or by special methods of injection. Rat. Seligmann and Jungeblut (160) explored the method of injection to the brain but got no differentiation among the types.

Mouse. Zinnemann (210), using the technique of intravenous injection to white mice determined the order of virulence to be *mitis* > *gravis* > *intermedius*.

Chinese Hamster. Tung and Zia (179) did not observe differences of pathogenicity amongst the types with this animal.

Spermophil. This animal was found more sensitive than most to diphtheria strains with the exception of the *intermedius* strain to which it was relatively resistant (39).

Method of Conjunctival Injection. Bieling and Oelrichs (10) using this method on guinea-pigs got the following results:

	Death	Perforation of Cornea
Gravis strains	5/15	8/22
Mitis strains	0/14	0/22

although in a second series of observations one *mitis* strain caused perforation.

TOXIN PRODUCTION BY THE DIFFERENT TYPES OF C. DIPHTHERIAE AND ON THE CONTROL BY STANDARD ANTITOXIN OF INFECTIONS WITH THESE TYPES

Soon after the *gravis* type of the *C. diphtheriae* was described it was shown by Parish, *et al.* (129, 130) that by comparison with *mitis* strains and much more so Park No. 8, the *gravis* strains were poor toxin-producers *in vitro* and that infections by them were adequately controlled by standard antitoxin. These facts are commonly accepted (84, 85), but they do not cover the whole case, for Murray (116) has shown that it is much more difficult to protect rabbits with standard antitoxin from repeated doses of *gravis* bacilli than from similar doses of the other forms. Then Gundel and König (67) have shown that if animals are actively immunized with various prophylactic agents, the best preparations protect against all strains, but the less satisfactory ones fail or partially fail to protect against *gravis* strains. Further, Gundel and Erzin (66) could successfully treat *mitis*-infected animals with standard antitoxin 24 hours after injection but not *gravis*- or *intermedius*-infected animals; and they showed that passive protection with antitoxin lasted considerably longer for *mitis* infection Povitzky, *et al.* (140) also showed in a small number of experiments that the control of *gravis* infection with standard antitoxin was more difficult than that of infections with other strains. Etris (43) demonstrated that *gravis* antitoxin was more potent than standard antitoxin in protecting animals against the homologous strain. Still more recently Clauberg (30), in an extensive study of toxins prepared in special media from cultures incubated only 24 hours, in which large numbers of *mitis* and *gravis* strains respectively were used, came to the conclusion that *gravis* toxins were on the average more potent in producing a skin reaction than *mitis* toxins and that their neutralization required more standard antitoxin.

One of the most interesting contributions to the subject and the most recent is that of O'Meara (121) who, working with saline extracts of *gravis* strains, was able to obtain a striking reinforcement and extension of the lesions produced by standard Park 8 toxin by combining sublethal doses of that toxin with the aqueous extract. On the basis of these observations, he supposes the existence of two elements in diphtheria toxin, one abundant in standard toxin tending to kill guinea-pigs in small amounts but producing only slight local lesions, the other slightly represented in standard toxin but abundant in the saline extract of young cultures on solid medium and tending to effect marked local lesions. It is not stated how far this second toxic element can be obtained from *mitis* cultures, and further investigation is necessary to elucidate this very interesting line of work. The observation that 1/50 of a normal M.L.D. of *gravis* strain kills when combined with a subnecrotic dose of staphylotoxin is interesting in this connection (158).

A new aspect of the problem of toxin production by diphtheria strains has been opened up by the work of the Massachusetts laboratories. Pappenheimer and Johnson (126) appear to have been the first to elucidate the role of iron in controlling toxin production as they were working with synthetic media and noted particularly the contrast between toxin production in flasks of soft glass and those of Pyrex glass. Happold (73) noticed that when grown in synthetic media with

a small and optimal iron content, a number of *gravis* strains produced toxin, although much less than the Park 8 strains, and that an interesting feature was that the former as well as *mitis* strains used up the iron content of the medium whereas the latter did not. Mueller (113, 114) has further pursued this work showing that, compared with the Park 8 strain and two others tested, the *gravis* strain was outstanding in its capacity to produce toxin in media rich in iron; and he points to the conclusion which may reasonably be deduced from this that in the conditions existing in the human body of marked iron excess the *gravis* strains may well prove to be the most potent toxin-producers. This is one of the most interesting developments of the study of toxin production by the different types, and it remains to be determined whether it will be found to hold when larger numbers of *gravis* and other strains have been brought under investigation.

All these observations suggest a complexity in the toxic product of diphtheria bacilli which yet awaits full elucidation and which may be specially difficult to define because one of the toxic elements tends to disappear in subcultures on artificial media whereas that studied in the standard preparation of toxin does not.

Feierabend and Schubert (45) in their study of the strains isolated at Prague in a severe epidemic 12 years ago drew attention to a type of strain which administered in doses that did not kill the guinea-pig produced a local lesion which was not controlled by antiserum. This property was lost, however, after repeated subculture. There are besides a considerable number of diverse observations which make it clear that the neutralization of toxin as usually prepared does not cover the whole problem of immunity in diphtheria. Thus on the one hand Parish observed a carrier of the diphtheria bacillus whose serum was completely void of antitoxin, whereas Prochazka (145) mentions a number of cases in which diphtheria had occurred in Schick-negative individuals with quite considerable amounts of antitoxin in their sera. Further, Seligmann and Jungeblut (160) were unable to control with antitoxin the infection of rats' brain which they could set up with C. *diphtheriae*. Lastly, Zoeller (214) discussing the disease as a clinical observer points out the importance of distinguishing between the qualities of receptivity for diphtheria strains and of capacity for reacting to diphtheria toxin.

CONSIDERATION OF THE TYPES IN THEIR SIGNIFICANCE AS EPIDEMIC STRAINS

There has been a tendency in many quarters to deprecate the use of the terms *gravis*, *mitis* and *intermedius* for the different types on the grounds that these terms are misleading since mild or trivial cases of *gravis* infection are observed on the one hand and severe and fatal *mitis* infections on the other (15, 36, 109). No claims that all *gravis* diphtheria is severe and all *mitis* diphtheria is mild have ever been made. The first considerable series of typed cases published (3) showed 144 mild cases of *gravis* infection and 5 severe *mitis* infections. It is true that no death occurred in the first 100 cases of *mitis* infection in that series. What was definitely suggested, however, was the similarity of the very severe clinical diphtheria occurring in Leeds at that time to that repeatedly recorded in Europe in the years immediately preceding; and that it was likely that the type of strain so closely associated with the Leeds epidemic would also be found in connection with the severer manifestations of diphtheria throughout the world; and that in this respect the name of "B. *diphtheriae Gravis*" would be found to be

justified. It was also claimed in this and subsequent publications that the toxic (myocardial, nervous, hemorrhagic) manifestations of diphtheria were specially associated with the *gravis* and *intermedius* strains, while the *mitis* strains were dangerous mainly from their obstructive effects in the air passages. How far has the much wider subsequent range of investigations justified these claims?

Relationship of Types to Epidemic Diphtheria and Severe Epidemic Diphtheria. The nature of an epidemic must and does vary with each infectious disease and it is difficult to find any complete analogy between one and another. The findings already described suggest that diphtheria, like enteric disease, is caused by several closely allied but distinct varieties of bacteria producing very similar but not altogether identical diseases. The spread of diseases of the upper respiratory tract, however, is much less tramelled by measures of sanitary control, and the development of epidemics is probably dependent in part at all events on fluctuations of mass immunity over periods of years. If this assumption is accepted then it would follow that whichever of the types of diphtheria bacillus possesses the greatest power of striking rapidly through the community will also prove to be the one showing the widest fluctuation in incidence, being practically absent in some areas and responsible for 90 per cent or upwards of the diphtheria in others. The figures for incidence of the different diphtheria types in various parts of the world definitely suggest that *gravis* is the type with greatest epidemic tendency and *mitis* that with least, while *intermedius* lies between. It would follow further from the conception of one variety as that with greatest tendency to epidemic spread that if cases are examined in sufficient numbers and in a wide enough area it would be found to be the preponderant type over all. *This also is the result obtained for gravis.* In a total of over 25,000 cases collected from all over the world, it is found to have been responsible for nearly twice as many cases as either of the two other types.

The picture of diptheria, as revealed by the study of type distribution, suggests therefore three closely allied conditions controlled to varying degrees by oscillations of mass immunity in the community, which are not synchronous in different areas; and possibly on all this there may be superimposed long-period cycles of change in the incidence of one or other of the types promoted by weather cycles in accordance with the suggestion of Wolter (198). This to the writer seems a much more probable surmise than that of the appearance of the *gravis* type as a modification of the *C. diphtheriae* not previously encountered.

Three separate cities, where diphtheria has been typed for several years, have each shown a phase of marked predominance of *gravis* infection at some period although these have not coincided in time in the different areas.

In Leeds a period of marked *gravis* predominance, high incidence of diphtheria and high diphtheria death rate has been followed by steady increase of *mitis* infection and a higher *mitis* death rate than previously, synchronising, however, with a general fall both in incidence of and mortality from diphtheria. In Liverpool where a diphtheria, in which at the outset all three types were well represented but *mitis* slightly predominated, was observed for three years there has been a sudden rise of *gravis* to a position of marked predominance at the end of the period of observation. This has been associated with an almost two-fold increase in the number of diphtheria deaths in the city. In Edinburgh, after several years

in which *intermedius* has played the chief part and *gravis* infection has been negligible, there has been in 1938 a marked increase in the *gravis* strains associated with a rise in diphtheria deaths but without any increase in diphtheria incidence.

There is one other area besides Edinburgh in which observations on type incidence have been made over a considerable period of years. These have not been sufficiently extensive and continuous to permit of presentation in the form of a curve. They are of considerable interest. It was shown by Murray (115) in Dundee that there as elsewhere in Scotland the predominating infection was *intermedius*, and *gravis* played a negligible part. The diphtheria death rate per 100,000 at Dundee at this time and for some years before and afterwards ranged from 3.4 to 9. In 1940, however, the incidence of diphtheria in Dundee increased to three times the average for the previous 10 years and the deaths per 100,000 rose to 41.8, a higher rate than in any year since 1890 with the exception of 1925, *i.e.* higher even than in some years of the pre-serum period. This explosive outburst was found by Tulloch to coincide with a gradual rise in *gravis* infection throughout 1940 till in the period December 17, 1940 to February 26, 1941, 47 cases examined showed 89% *gravis*, 13% *intermedius* and no *mitis*. There are a number of other areas in which observations of the incidence of diphtheria types have been made and in which there has been a record of severe diphtheria epi-

TABLE 9

Gravis incidence in areas in which severe epidemic diphtheria has been recorded

AREA	TIME OF OBSERVATION OF TYPE INCIDENCE	PER CENT OF GRAVIS INFECTION	APEX YEAR OF EPIDEMIC	DIPHTHERIA DEATHS PER 100,000 OF POPULATION IN THAT YEAR	REF.
Hull.................	Nov. 1932–Feb. 1933	59	1932	42	(98)
Warrington..........	1934	90	1934	49	(107)
Berlin...............	1933	77	1927–29	13	(22)
	1934	78	1927–29	13	(155)
Kharkoff	Oct. 1936–Aug. 1937	72	Aug. 1936	*	(211)
Stadt Brandenburg..	1935	95	1935	*	(64)
Cork...............	1934–35	91	1930	80	(35)

* Not available.

demics at the time of observation or some years beforehand. The incidence of *gravis* diphtheria in these areas is recorded in table 9.

The associated diphtheria mortality per 100,000 of population is undoubtedly the safest criterion of the menace to the community exerted by any given strain of bacterium, and when the figures for Cork, Warrington, Hull, Dundee and Leeds are compared with (a) that for 88 of the chief cities of America in 1937, *i.e.* 1.46, or (b) the average for England and Wales for 10 years (1929–38), *i.e.* 7.9, it is clear that these places have suffered from epidemics of a very severe order. In all except Hull and Cork, the incidence of *gravis* at the height of the epidemic was about 90% of all diphtheria. In Cork no observations were made at the height of the epidemic, but in view of the persisting 90% *gravis* infection several years later and of similar observations (129) on a small series of strains taken earlier than 1934, it seems altogether probable that this strain was mainly responsible. In Hull where the observations were made as the epidemic commenced to decline, the incidence of *gravis* was only 59% but as in these cases a 19% case mortality was recorded, it again seems most probable that they were responsible for the epidemic.

In Liverpool, where *gravis* has played a considerable and increasing part in the diphtheria of the city since observations on type incidence were started, this strain has developed an epidemic tendency within the last year which has been reflected in the marked rise in the curve of mortality in 1941.

In Berlin the diphtheria mortality per 100,000 has never approached that recorded in some of these smaller centres and this notwithstanding the fact that it was more than quadrupled between 1924 and 1927 (49), and that the extreme gravity of many of the cases (38) has caused much consternation. Here also it seems probable that a *gravis* strain has been responsible although no observations on the types of diphtheria bacilli were undertaken when the epidemic was at its height.

It may well be, however, that a very sharp epidemic passes more slowly through a larger community like Berlin and is waning in one part of the city while rising in another. In such a case the rising mortality in some areas would be partly balanced by falling mortality in others, and an epidemic plateau would be produced as in Berlin in 1927-28-29 instead of a sharp epidemic spike as in Leeds, Hull or Dundee. However that may be and in spite of a higher case mortality rate for *intermedius* than for *gravis* in a number of areas (Liverpool, Romford and Manchester, in 1933), no record has yet been obtained of an area in which *intermedius* has been the predominant strain during a period of epidemic incidence of diphtheria or of high death rate per 100,000 of population (18, 24, 34, 58, 63, 76, 90, 115, 149). In those instances in which figures for death rate per 100,000 in the areas concerned at the time of observation are available, they vary from 14 in Glasgow in 1934 to 3 in Dundee in 1934 and Edinburgh in 1935.

In Stafford (106), in Newcastle (35), in Maryland (137), in Capetown (206), in Amsterdam (165), and in Moscow (163) observers describe diphtheria in which *mitis* is the predominant strain and the diphtheria is definitely mild. In Magdeburg (78), in Lemberg (215), in Halle (122) and in Hamburg (185) diphtheria is also described in which *mitis* is the most numerous train; but no mention is made of any specially severe associated epidemic diphtheria, though Magdeburg had an unusually high case death rate for *mitis* diphtheria which has ready been discussed. In so far as they are available, the death rates per 100,000 of population in these areas with *mitis* diphtheria, the most numerous, range from 0.6 in Amsterdam in 1935 to 8 in Newcastle in 1934.

GENERAL SUMMARY

The existence of three well-defined cultural types of the diphtheria bacillus for which the designations *gravis*, *intermedius* and *mitis* have been suggested has been widely recognized. There are, however, a small percentage of strains which do not correspond closely to any one of these types. The proportion of these atypical strains varies from place to place and is highest where the diphtheria is mild or of moderate severity. The atypical strains are more often found among carriers and convalescents but have more rarely been found to be associated with severe and even fatal illness. They have not been observed at any time to develop an epidemic tendency. The *mitis* strains when they cause death do so mostly in infants owing to obstructive phenomena and pneumonic complications. The *intermedius* strains are very near to the *gravis* strains in the severity of the clinical conditions which they produce although over all the associated case death rate is less than with *gravis* infection and has never reached quite such a high level as the latter in its most severe manifestations. There are, however, many areas in which the *intermedius* case death rate exceeds that due to *gravis*. The *intermedius* strain disappears more rapidly in convalescence, and whether on account of this or for other reasons it has not the same tendency to epidemic spread as *gravis* infection. The outstanding clinical features of severe *intermedius* and *gravis* diphtheria are essentially toxic and hemorrhagic phenomena, myocardial weakness and pareses. The *gravis* strain is outstanding on account of a greater and more constant pathogenicity to animals, a deeper penetration of the tissues in

the human body and a greater epidemic potency. A complete explanation of these points of difference is still lacking.

It seems on many grounds probable that the numerous severe outbreaks of diphtheria described in Central Europe in 1927–37 and found to be specially intractable to serum treatment were due to the *gravis* type of diphtheria bacillus. The question whether the brilliant results of prophylactic inoculation recorded in North America owe their superiority to those obtained in Europe to more comprehensive adoption and better execution or to the absence of epidemic *gravis* diphtheria in the former area still remains to be determined, although the value of this procedure over all has been proved beyond dispute.

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THE SIGNIFICANCE OF THE Vi ANTIGEN

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Typhoid fever is still a public health problem of considerable importance in many parts of the world. Any contribution to our fund of knowledge concerning the etiologic agent (applicable to diagnosis, prophylaxis, and therapy) is, therefore, to be welcomed as a possible basis for better control of the disease. The discovery by Felix and Pitt in 1934 (30, 31) of a hitherto undescribed antigenic component of the organism responsible for the disease was a step in this direction. Up to that time *Eberthella typhosa* was known to contain at least two heat-stable antigens in the somatic fraction and one specific antigen in the flagellar fraction. The toxic manifestations of typhoid fever were commonly thought to be due to the endotoxins contained in the somatic fraction. An additional property of the organisms, virulence (at least for mice), was now described as being associated with the presence of the new-found component, and it was accordingly named "Vi". It was this concept of the antigen being a virulence antigen which gave such pronounced impetus to renewed study of the typhoid organism; for the implications concerning severity of attack and immunity were quickly seen. The purpose of this review is to take bearings on how much progress has been made as a result of the studies thus stimulated, and to call attention to places where a strengthening of evidence would be desirable.

Occurrence. Almost all freshly isolated typhoid cultures have been shown to contain the Vi antigen (29, 46, 20, 1, 66, 57). In addition, it has been found sporadically in some of the Hirschfeld paratyphoid C strains (46), in a species of Salmonella designated as "ballerup" (49), and in a few cultures of the coli group (48).

Strains of *Eberthella typhosa* which possess the antigen do not differ noticeably in cultural reactions (including fermentative ability) from those which lack it, although Giovanardi (36) reports that colonies of the Vi-containing form are somewhat more opaque than those of the forms not containing it.

Properties. The antigen has been precipitated from protein-free trichloracetic acid extracts by uranyl salts (13, 59). Freeman and Anderson (34) were unable to repeat this separation from the O complex and hence are not convinced that the Vi properties may be ascribed to a compound separable from the O fraction. However, Henderson (43) demonstrated at least part of the Vi substance free from any of the smooth O substance in anhydrous diethylene glycol extracts of rough Vi-containing forms.

Acetone extracts containing Vi and O fractions show a precipitation reaction with phosphotungstic acid in 5% sulphuric acid and with neutral lead acetate, uranium acetate, mercuric acetate and aluminum sulphate, whereas extracts containing only the O fraction fail to show precipitates with these reagents (65).

The antigen is probably situated for the most part near the surface of the

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organisms (11). Its presence causes inhibition of O agglutination, even though the organisms thus inhibited contain the O antigens. It is spontaneously unstable; mere storage, even at ice-box temperatures, will result, in a matter of a few weeks, in loss of at least some of the properties ascribed to the antigen. Heat, and the action of most chemicals which have been tried hasten this destruction. Only mercuric chloride, formaldehyde, acetone, and ethyl and methyl alcohols seem, among those investigated, to be weak in destructive effect (11, 35, 2). According to Peluff (55), the preserving effect of the alcohols and acetone on the Vi component is probably due to their dehydrating action. Cultures suspended in absolute alcohol or acetone were found to retain Vi characteristics even on heating. Dehydration by evaporation has also been found to preserve Vi characteristics.

In this connection it might be mentioned that the method of detecting destruction of the antigen is important; not all of its properties disappear simultaneously. Inhibition of O agglutinability is the first property associated with the presence of Vi to be lost. Hence acquisition of O agglutinability of a culture previously inagglutinable is one of the most sensitive methods of detecting alteration of the Vi fraction. Other manifestations of the presence of Vi in suspensions of organisms disappear in the following order: *1*, ability to stimulate the production of agglutinins in measurable amounts in rabbits; *2*, ability to agglutinate in Vi tiserum; *3*, ability to absorb Vi antibodies from an antiserum. In other words, suspension seemingly devoid of Vi antigen as determined by the usual criteria may still be capable of absorbing appreciable amounts of Vi agglutinin from a specific antiserum. In the work on the effect of chemicals cited above, the criteria for destruction generally used were inability to stimulate the production of agglutinins or inagglutinability in Vi antiserum.

Some of the properties of Vi-containing cultures described above as being labile when subjected to the action of heat or chemicals may also be found to be altered simply in the course of routine transfer of the cultures. For example, cultures which on original isolation are entirely inagglutinable in O antisera very often acquire O agglutinability after one or more transfers although they may still be agglutinable in Vi antisera as well and may be capable of stimulating Vi antibody production. Cultures of this kind, which have gained O agglutinability but still possess Vi *agglutinability* may, in the course of subsequent transfers, lose all evidence of the latter. *Certain deleterious factors in the environment of the growing organisms may hasten these changes.* Kauffmann (46) has developed a terminology for these cultural changes. *Those organisms which contain Vi antigen and are inagglutinable in O antiserum are termed V forms, the V standing for "viel".* When they have gained O agglutinability but still retain Vi characteristics they are V-W forms (transition). When they have lost all Vi characteristics, as determined by ordinary tests, they are called W forms (from "wenig"). The change from V to W involves loss of an antigen and hence is termed V-W degradation. Kauffmann believes that even W forms retain Vi potentialities and that under certain conditions, though admittedly

rarely, they may regain Vi characteristics. He has cited one instance of such a reversion in culture H901 (a W culture) growing in a mouse.

The question of multiplicity of the Vi antigen. Considerations such as the previously described fractional destruction of the antigen have prompted some investigators to postulate a multiple nature for it. Some of the considerations, in particular, are that treatment with formaldehyde has been shown (28) to alter the ability of a Vi-containing bacterial suspension to produce protective antibodies while leaving its agglutinogenic and precipitinogenic capacities intact. Likewise, extracts made with diethylene glycol have been shown (44) to stimulate agglutinin and precipitin production but not protective antibodies in like degree. Conversely, heat-killed suspensions do not produce agglutinins in appreciable amounts but serve very well for the production of protective antibodies (31). These observations have led Craigie and Brandon (18) to assume a complex nature for the "Vi" as originally described. For the agglutinogenic part of the complex they use the symbol "V", borrowed from Kauffmann (46), assuming that the portion which stimulates agglutinin and precipitin production is different from that which stimulates protective antibodies. The symbol, "Vi", they reserve for the entire complex. That differences in activity exist between suspensions and extracts prepared in different ways cannot be gainsaid. But that the differences are always manifest along the line of separation of agglutinin production and protective antibody production has not been adequately proven. In this paper, therefore, only one symbol, Vi, will be used; and it will be intended to designate any or all of the aspects of the antigen.

The most logical explanation for the differences in activity among the various chemically treated suspensions and solutions seems to be the hypothesis favored by Henderson and Morgan (44) of a single compound which may be altered physically, chemically and antigenically by various manipulations. The possibility of a single compound possessed of two or more antigenic radicles might also be investigated. Chemical characterization as complete as that which has been accomplished for the pneumococcal polysaccharides has not been reported for this fraction.

The differences among Vi-containing strains revealed by the bacteriophage studies of Craigie and Yen (21, 22) would seem to be significant in this connection. To the present, no means of detecting these differences other than the action of the phages has succeeded, and the adaptive variants of the single phage used as reagents for this purpose have shown no serological differences. Yet the evidence shows that the differences among the bacterial strains are hereditary and stable, and are therefore real differences which should at some future date yield to other methods of analysis.

Relation to virulence. The center of the entire discussion concerning this antigen has been the question of its relationship to virulence. The original tenet of Felix and Pitt (31) was that the presence of the Vi antigen rendered organisms resistant to the action of O antibodies and hence made them better able to invade a host which they were infecting. In mice, this power was sup-

posedly responsible for the higher death rate of those animals injected with a standard number of Vi-containing organisms than those injected with the same number of organisms lacking the Vi antigen. Further work (47, 23, 2) has substantiated a qualitative relationship between the presence of this antigenic component in smooth typhoid cultures and their ability to kill one or more mice injected intraperitoneally with a standard dose (about 100 million organisms when administered without mucin). With rough, typhoid organisms (32) or with paratyphoid C organisms containing the antigen (47) this relationship does not hold. Seemingly, then, virulence for mice is not a function of the Vi antigen alone, but of a combined action of the Vi and O antigens of the typhoid organism.

The mechanism of this enhanced mouse-killing ability when Vi antigen is present has not been established. The increased invasive power, believed by Felix and Pitt to be responsible, has not been actually demonstrated. The attempt of Ørskov and Kauffmann (54) in this direction demonstrated, rather, that there was no difference either in invasive ability or survival between Vi and non-Vi forms, when mice were infected with sub-lethal doses. Grasset and Lewin (39) found even less invasion of the blood stream by Vi than by non-Vi forms when larger doses were used. Both of these teams of workers have therefore postulated greater toxicity for the Vi-containing form, assuming that large doses of the organisms containing this toxin so weaken the animals as to break down the normal inhibitory mechanisms and permit their being overwhelmed. Since most efforts to demonstrate higher toxicity for these cultures have failed, this hypothesis, too, remains unsubstantiated. Felix and Pitt showed in their earliest work (30) that killed suspensions of "virulent" organisms were not more toxic than "non-virulent" cultures. Topley and co-workers (65) found that alcohol precipitates of acetone extracts were not more toxic when derived from Vi cultures than from non-Vi cultures. Smith (64) found the toxicity of aqueous extracts for mice and rabbits to be independent of the Vi character of the organisms. And yet all of these workers demonstrated by serological means (either by protection tests, or by titration of homologous antisera) that the Vi character remained intact in their preparations. Boivin and Mesrobeaunu (12, 13), after having separated the Vi and O fractions by chemical means and having confirmed the identity of these preparations by immunological tests, found that it took five times as much of a Vi preparation as of an O preparation from the same culture to produce a given toxic effect in mice, and that the symptoms of intoxication with either fraction were the same. The only data which might be interpreted as supporting the hypothesis of higher toxicity of Vi cultures are those of Aoki (5) who claimed a higher toxic effect in mice for heated aqueous extracts of virulent cultures than for similar extracts of non-virulent cultures. Since he did not attempt to demonstrate the Vi fraction in these cultures or extracts, however, his results cannot be properly evaluated.

The evidence thus far summarized indicates only that there is some obscure synergism between the Vi and the smooth somatic antigens of the typhoid organism which is responsible for killing mice, but not for the invasion of the animals

when sub-lethal doses are injected.   A possibility which has not been adequately investigated is that of superior multiplication (as differentiated from invasion) of the Vi organisms in comparison with non-Vi in mice, resulting finally in death by intoxication owing to the large number of organisms present.   The experiments of Ørskov and Kauffmann cited above are not sufficiently discriminating to stand as evidence against this possibility.   The doses which they employed were of such proportions that the mice exhibited no intoxication as a result of the injections; in other words, the natural immunity of the animals sufficed to prevent signs of disease.   Under such conditions Vi and non-Vi forms were found to be possessed of the same order of invasiveness and to survive for the same lengths of time.   The invasiveness, or at least the capacity to multiply to a point which would result in death by toxemia, might be quite different if the initial dose were of such magnitude that the natural defenses could not cope with it, and the deciding factor in failure of the natural immune forces to cope with a given sized dose might conceivably be the Vi substance situated near the surface of the organisms.   The work of Bhatnagar (9) showing that Vi-containing organisms are less easily phagocytized than non-Vi is a case in point.

Role of Vi antigen in human infection.   In human typhoid infection we are not ordinarily confronted with a rapid lethal effect but rather with the survival of organisms within the host for protracted periods.   In contrast to the case in the mouse, this protracted survival is accompanied by signs of disease.   The mechanism of virulence for man may, therefore, not be the same as that for mice.

All studies to date indicate that human infection is almost always brought about by cultures containing Vi antigen (29, 46, 20, 1, 66, 57).   The exceptional cases in which Vi antigen was not demonstrated in cultures isolated from cases and carriers cannot be cited as evidence of infection without the presence of this antigen, since no systematic attempts to isolate other cultures from the same patients were made.   It has been pointed out (20) that a "V" bacteriophage can be isolated from many of these cases, and (3) that random sampling from plate cultures—the method usually employed for isolating these organisms—is likely to yield occasional non-Vi forms even when other colonies on the plate are Vi in character.   Thus, the questions, whether human typhoid fever is ever caused by organisms preponderately non-Vi, and whether such infection, if it occurs, is less severe than that usually encountered, are entirely unanswered.

Among the cases in which the clinical record was studied along with the bacteriological findings, no correlation could be shown to exist between the degree of development of Vi antigen in the cultures isolated and the severity of the disease (29, 58, 1, 57).   However, there is no satisfactory method in use for evaluating the quantitative development of the Vi antigen, and the problem is further complicated by differences in immunological response among individuals, which is also difficult to measure.   Titration of antibodies, particularly Vi antibody, has been attempted, but leaves much to be desired.   The results give only a confused picture, possibly because titratable circulating antibody is a very poor index of immunological response.   Felix, Krikorian, and Reitler (29), Almon and Stovall

(3), and Almon, Read and Stovall (1), found Vi antibody to a titratable degree in distinctly less than half the cases of typhoid fever investigated, and found no correlation between the stage or symptoms of the disease and the presence of the antibody. The differences in severity of attack or promptness of recovery have not, therefore, been demonstrated to be due to the presence or absence of the Vi agglutinin.

The question of human infection cannot be dismissed without mention of the bacteriophage or bacteriophages active against Vi-containing organisms, first mentioned in 1936 (18, 60, 62). Phages of this kind have been shown to have the capacity, when experimentally introduced, of destroying typhoid organisms *in vivo*, in mice (7, 69) and in humans (68). Whether this action ever occurs outside of the experimental realm is not known. The consideration adds just one more factor to the complexity of the problem of immunity.

What role the Vi agglutinin plays in the carrier state is not known, although its presence has been demonstrated in the sera of so high a proportion of chronic carriers that titration for it has been recommended as an adjunct to the demonstration of organisms in stool and urine in finding hitherto unrecognized carriers. Among those who have shown the agglutinin to be present in a high proportion of carriers are Pijper and Crocker (56), Bhatnagar, Speechly, and Singh (11), Felix (27), Almon and Stovall (4), Eliot (24), Eliot and Cameron (25), and Coleman (16). Demonstration of the antibody in chronic carriers would seem to indicate that it plays no superior role in ridding the body of organisms,—the organisms co-exist with the antibody in the carrier state for indefinite periods.

Mouse protection. The knowledge that the Vi component may be studied as an autonomous antigen has naturally led to experimental studies particularly concerned with the role of its corresponding antibody or antibodies in the protection of mice,—those animals for which virulence has been demonstrated to be associated with the antigen. A large part of the literature on the general subject in hand deals with this aspect, and the results have been made the basis for recommendations concerning the production of vaccines and antisera for human use.

The earlier studies concerning the protection of mice immunized either by the injection of organisms or the injection of antisera indicated that protection against strains virulent for mice was generally better if the immunizing suspension was also a virulent one, or if the antiserum administered contained Vi antibodies; but they also indicated that these antigens and antisera did not afford superior protection against lethal doses of organisms low in virulence. (See Felix and Pitt (31, 32), Brown (15), Kauffmann (47), Ørskov and Kauffmann (54), Grasset and Lewin (40), Army Medical School (6).) It was further shown, however, that repeated immunization with non-Vi cultures could protect against several lethal doses of Vi-containing cultures (65). In an experiment by Norton and Dingle (53), the test dose was given intracerebrally and could therefore be better controlled as to the number of organisms since smaller numbers sufficed. Under these conditions, differences in protective action between anti-

sera prepared from virulent organisms and those prepared from cultures very low in virulence could not be shown. This is indicative of the general trend of more recent work: in instances in which better control is exercised, the importance of the Vi antibody as a qualitative factor is less striking. These results and the result above mentioned in which Vi cultures and antisera failed to protect against non-Vi infection (in spite of the presence of O antibodies) seemed anomalous until the entire matter of mouse protection was shown by Henderson (42) to be a function of the amount of antibody present as related to the number of organisms used in the test dose. The protective antibodies were contained in rabbit antisera used in passive protection experiments on mice; and in these experiments either Vi or O antibody was found to be effective in preventing death of the animals, though against Vi-containing organisms, the Vi antibody was relatively more efficient. Results reported by others which previously seemed contradictory fall nicely into line when interpreted in this light, and provide added evidence for the validity of the observations.

What seems to be significant in all of the work cited above is that the Vi antibody carries out no function that the O antibody alone cannot fulfill, although, against Vi-containing organisms the Vi antibody frequently acts with greater efficiency. This superior efficiency may be due to quantitative rather than qualitative considerations. The measurement of antibodies in the work of Henderson was based upon titration. Amplification to include determinations of antibody nitrogen by the method of Heidelberger and Kendall (41) should be carried out in order to evaluate the quantitative factor; for the low titers of Vi antibody usually evinced are not necessarily indication that the amount of antibody present is small. Not until such chemical studies have been carried out can the matter of the importance of the Vi antibody as an entity serving functions different from those of the O antibodies receive proper evaluation.

Active and passive immunization of man to the Vi antigen. There is no incontrovertible evidence that the Vi antibody performs any function in mice which the O antibodies cannot perform. It has nevertheless been assumed that Vi antibody is desirable for prophylaxis in man and an adjunct in the curative process.

What evidence we have from the occurrence of the antibody in natural infection in man lends no support to the validity of the above assumption. In the discussion in a previous section (Role of Vi antigen in human infection), it was brought out that a special function of the antibody as it occurs naturally has not been demonstrated. Moreover, the artificial administration of this antibody during active cases has been so inadequately controlled as to contribute nothing to the argument. Improvement in severe cases, some of them seemingly moribund, has been described following administration of horse serum containing Vi and O antibodies (26, 51, 52), but no control cases were treated with sera containing only the O antibodies. This lack of evidence does not necessarily invalidate the assumption of an important role for the Vi in human infection; it merely points up the need for further work. It should be borne in mind in future studies that it is not only possible but likely that the relationship between the amount of antibody and the number of infecting organisms, demonstrated

by Henderson (42) to be the important factor in the survival of mice, will be important also in human infection.

Although there is no experimental or statistical evidence which will bear scrutiny showing the Vi antibody to be important in preventing or combating human typhoid fever, the general feeling that suspensions to be used for human vaccination should be prepared from cultures containing the antigen may be sound. This feeling is based on the realization that: 1, typhoid vaccine will bear improvement, 2, almost all human typhoid fever, if not all, is caused by Vi-containing organisms, and 3, the Vi antibody has been found superior to O antibody in protecting mice against the lethal effect of Vi-containing cultures. The work in support of the last two points has been discussed in previous sections. The arguments concerning the possibility for improvement in typhoid vaccines are brought out chiefly in the book: "Immunization to Typhoid Fever" from the Research Laboratories of the Army Medical School. Analysis of the tables contained therein shows that vaccination with heat-killed suspensions of the old Rawlings strain, known now to be poor in Vi antigen if not devoid of it, served to reduce the incidence of typhoid fever considerably below that for unvaccinated groups under conditions of poor sanitation. These same tables show, however, that there is room for improvement in that the incidence of the disease among vaccinated groups is always higher where sanitation is poor (i. e., rate of exposure high) than among unvaccinated groups living in an environment in which the sanitary factor is satisfactorily controlled and hence the rate of exposure is low.

The U. S. Army is now preparing its typhoid vaccine from a culture which, by a number of criteria, has been shown to be superior in immunizing qualities to other cultures hitherto used. Titers for O antibody produced by it are among the highest, and titratable O antibodies persist for longer periods in human subjects vaccinated with it than in those vaccinated with other cultures. Experiments in active and passive immunization of mice likewise demonstrate the superiority of this culture. It has been shown to contain the Vi antigen, but studies of the corresponding antibody have not been reported,—unfortunately for the purpose of this review. It is possible that the use of a vaccine of this nature will result in the production of some Vi antibody, even though heat-killed suspensions be used; for there is a growing body of evidence that heat-killed vaccines may give rise to Vi agglutinin,—titratable during a fleeting period.

Early work with heat-killed cultures failed to demonstrate Vi agglutinin production in rabbits, but with refinement in methods of detecting the antibody we have found it in some of our rabbit antisera, and Horgan (45) reports it also. Early attempts to demonstrate the antibody in human sera following vaccination likewise failed (10, 24); but unpublished results of work at this laboratory have shown that if blood is drawn at the right time, such vaccinated individuals may sometimes reveal it. Weekly studies made on the sera of five young women showed that three of them had developed Vi agglutinins in demonstrable titer (1:40 or higher) at the third, fourth, and fifth week after the last injection of vaccine. In each of these cases the titer of Vi antibody reached its highest point just when the titer of H antibody began to drop. The titer of Vi antibody

dropped rapidly after that, and demonstrable correlation with the O antibody did not exist. Coleman (16) has also found the antibody in a higher proportion of vaccinated individuals than in the general population by the use of an agglutination technique which involves centrifuging. Results from mouse protection experiments (28) indicate that protection may not be correlated with the presence of agglutinins. Almost no experiments on the passive protection of mice by human antisera evolved by different methods have been carried out. The work of the Research Laboratories of the Army Medical School (6) cited above was along this line, but the report contains no information on the Vi agglutinin content of the sera used. Hence, if the present vaccine in use by the Army proves to be superior to the old, the responsibility of its Vi antigen content cannot be gainsaid; neither, however, can it be considered unequivocally proved.

One of the few efforts to prepare a vaccine with the avowed purpose of stimulating Vi antibodies in man is that of Felix, Rainsford, and Stokes (33) who have prepared an alcohol-killed, alcohol-preserved suspension which gave rise to Vi antibody in demonstrable titers in 30% of 84 individuals receiving it, as contrasted with 5.6% of 228 individuals receiving heat- or alcohol-killed suspensions preserved with phenol.

It would not be wise to leave the subject of the immunization of man against typhoid fever without some mention of other methods of preparing the immunizing material. In the "endotoxoid vaccine" of Grasset (37, 38) there is a preparation the superiority of which seems to be fairly well supported by statistical data. The use of this preparation among the personnel of the mines of South Africa, numbering upwards of 400,000 people, has given results better than the use of the standard type of vaccine, if the statistics can be relied upon. This preparation has not attracted the attention which these results seem to warrant. But again we are at a loss concerning the role which the Vi antigen may be playing, for whether such an endotoxoid gives rise to Vi antibodies has not been investigated. It is known that the cultures from which it has been prepared have been Vi-containing cultures, and that in mice such preparations had a better protective effect than similar preparations from strains lacking in Vi (39).

The suspension killed with mercuric chloride described by Bartos and Buchgraber (8) also deserves further investigation inasmuch as less local and systemic reaction than with the conventional preparation is claimed for its use, and inasmuch as it has been shown by other workers (see section on "Properties") that mercuric chloride used as the killing agent permits the preservation of the Vi antigen. The concentration of mercuric chloride (.02%) is so low as to entail little danger of toxic effects in the human subject with the doses ordinarily used.

Laboratory applications. An evaluation of the significance of the Vi antigen would not be complete without mention of its utilization as a reagent in diagnostic laboratories. Its application in locating chronic typhoid carriers was mentioned in the section on "Role of Vi antigen in human infection". This application has obtained a toe hold in standard laboratory procedure through the recommendation of the Committee on Diagnostic Procedures and Reagents

of the American Public Health Association (17). In addition, rapid methods for the identification by means of Vi bacteriophage, not only of the typhoid organism but of different phage-susceptible strains, have been developed by Craigie and Brandon (19) and Craigie and Yen (21) and have been shown by these and other workers to be applicable to the analysis of epidemics (22, 14, 67, 50).

SUMMARY

The Vi antigen is contained in the carbohydrate fraction of the typhoid organism and has been separated from other antigenic parts of this fraction, but has not been characterized. Since certain chemical treatments result in the loss of some of its properties while leaving others intact, a complex or multiple nature seems indicated.

The antigen, in the presence of the O antigens of the typhoid organism, provides enhanced "virulence" for mice, in the sense that smaller sized doses suffice to kill these animals when Vi antigen is present. The mechanism by which this enhanced killing ability is brought about has not been demonstrated experimentally. All the evidence indicates that it is not due to greater toxicity of the organisms containing the fraction. It is suggested that these organisms may multiply faster than those lacking Vi antigen due to the protective action of this antigen against phagocytosis.

Almost all typhoid organisms possess the antigen when freshly isolated from carriers and cases. Since, then, there are no cases proven to be due to organisms lacking Vi antigen, the establishment of a function for this antigen in human infection suffers from want (for purposes of comparison) of cases in which it is absent. Inability to make satisfactory quantitative measurements of the degree of development of Vi antigen in any given culture adds to the difficulty in interpreting its function.

Vi antibody is a somewhat more efficient agent for protecting mice against the lethal effect of Vi-containing organisms than are the O antibodies. It is not know whether the explanation for this is a different action of the Vi antibody against the test-dose organisms, or whether the amount of Vi antibody in the sera used for these experiments has exceeded the O antibodies in the sera used for comparison. No quantitative studies on antibody nitrogen have been carried out on these sera. Where comparisons by titer have been possible (and this is possible only when comparing different sera containing the same kind of antibody), the protective efficiency in mice has been shown to be a function of the amount of antibody present relative to the number of organisms in the test dose.

Although there is no good experimental evidence to show the necessity for or efficacy of the Vi antibody in combating human infection, it would seem, since most, if not all, human typhoid infection is due to Vi-containing organisms, that the corresponding antibody along with O antibodies might be valuable in protecting populations; and efforts are widespread to improve vaccines accordingly. Most of these efforts have failed to measure the Vi antibody response in human

subjects but have been based, instead, on the virulence and protective qualities in mice for the cultures employed.

The titration of suspects' sera for Vi antibody is gaining recognition as a valuable adjunct to stool and urine cultures in the search for typhoid carriers, since a high percentage of carriers have been shown to have demonstrable amounts of it, and since vaccinated individuals, if they show it at all, show it only transitorily.

The multiplicity of Vi bacteriophages and correspondingly sensitive strains of the typhoid organism has found application in the analysis of epidemics.

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STATISTICAL METHODS AND CONTROL IN BACTERIOLOGY[1]

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CONTENTS

It has been alleged that certain people use statistics as a drunk does a lamppost—more for support than illumination. Such a criticism of the misuse of statistical methods is unfortunately too often justified and probably is the basis of the somewhat passive but nevertheless widespread opposition encountered when these methods are first introduced into any biological discipline. That this first phase of more or less passive resistance is about completed in bacteriology is evidenced by the ever-increasing references to statistical treatment of the data in its journals. Not so very long ago statistics touched bacteriology primarily in just one field—bacterial enumeration—but current literature provides statistical analysis of data from diverse experiments. As examples the following are cited: virulence of streptococci for mice (10); relation between growth of bacteria and the heat stability of their enzymes (25); deterioration of cellulose fibers by fungi (43); reliability of soil counts (54, 55); disinfection of trout eggs (39); test of fungicides on mold spores (111); probability of isolating a pure culture in a Petri dish (68). These applications, with many others which will be described in greater detail in the text, suggest

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that the time is appropriate for a review of the use of statistical tests and control in bacteriological work.

This paper is somewhat different from that usually published in the BacTERIOLOGICAL REVIEWS in that the literature serves primarily as a source of more or less familiar examples useful for illustrating the statistical principles discussed. Thus, the intrinsic significance or insignificance of the data selected for purposes of illustration is beside the point. The extension to data which differ in content but not in the principles involved should not be too difficult and "is an exercise left for the reader."

Part I deals with the distributions of variables; application of the knowledge of different types of distribution is illustrated by examples of statistical control of laboratory procedures. The important point in this section is an appreciation of the principles rather than mastery of the details of the calculations. In contrast, the arithmetic of the statistical tests of significance discussed in Part II is given in some detail since this facilitates an understanding of their application to actual data. Although, at first sight, some of the problems solved in this part may appear rather complicated and the calculations formidable, close examination will demonstrate that they involve easily-followed procedures. In passing, it should be noted that the titles of these major sections refer to the primary emphasis in that portion of the paper, but the subject matter is not entirely restricted to that implied by the section heading. Thus, some tests of significance are necessarily used in Part I in order to illustrate certain aspects of the distributions of variables. Likewise, the various statistics discussed in Part II have other functions, e.g., for description of data, as important as is their use for the statistical tests. In both Parts I and II it has been necessary to introduce certain technical terms which are precisely defined only in mathematical terminology. To maintain continuity of both style and content in the text such terms are used without comment and are defined and discussed in the Appendix.

Finally, it is emphasized that this paper is not intended as a course in statistics. The mathematical formulation has been kept to a reasonable minimum and emphasis placed on mathematical assumptions and principles of statistical theory as they relate to statistical interpretation of experimental results. Without special knowledge, it is often difficult to say whether these assumptions obtain in a given body of experimental data. Blind application of the formulae may therefore lead to error. This pitfall is best avoided by seeking the aid of a qualified statistician. Except for a few simple applications, such as determining means or slopes of lines, the biologist should ordinarily no more attempt the statistical analysis of his complicated experimental findings without consulting the trained specialist than he should try to analyze his cultures for an isotope without the advice of a physicist. It is natural for the biologist to ask: "Then why should I know anything about the subject?" Primarily, to recognize problems in his research which might benefit through statistical interpretation or control. Of almost equal importance, to be sufficiently informed so that he can present his material intelligently and concisely to the statistical consultant.

Statistical Control. In the foregoing the term, *statistical control*, has been used frequently and a more detailed exposition of what is implied in this phrase may be of value. The importance of statistical control of laboratory and field techniques needs greater emphasis in biological literature. Laboratories using statistics have employed almost exclusively *tests of significance* and have neglected the opportunity afforded for day-to-day check on the reliability of a routine analytical method. An experimental procedure is said to be in a state of statistical control when the observations to which it gives rise under what are assumed to be 'essentially the same conditions" fluctuate in a random manner and are free from trends and non-random shifts in magnitude. Unless a sampling procedure—and in a sense all processes leading to observations *are* sampling procedures—is in a state of statistical control, it is not possible to make valid inferences about the 'population' which the observations are supposed to represent. In the important paper by Fisher, Thornton and Mackenzie (34) this is stated: "Any significant departure from the theoretical distribution is a sign that the mean may be wholly unreliable."

In science, industry, and commerce where decisions must be made on the basis of results of some series of measurements, the reliability of the methods must be known. To know that the methods provide good checks or even that two operators obtain similar results is not enough. It is generally recognized that in arguing from the particular to the general the wrong decision will occasionally be recommended by the observations obtained, and we should know how frequently these errors are apt to occur. If too often (in industry, the economic consequences furnish a valuable measure of how frequent is 'too often'), then the procedure must be altered so as to reduce the expectancy of false decisions. When a procedure is statistically controlled, the expectancy of a false decision is a minimum. The Western Electric Company (equipment manufacturer for the Bell Telephone System) has led the way in applying statistical control to manufacturing processes, and other large industrial organizations (*e.g.*, General Electric Company, United States Steel Corporation) have found it profitable to follow suit.[2] The statistical staff of the Rothamsted Experimental Station in England has pioneered in showing biologists how to check their sampling techniques and has stressed the importance of doing so.

More evidence regarding statistical control might well be included when publishing research, since its absence may modify conclusions profoundly. When a series of observations exhibits properties widely divergent from those characteristic of random samples of a hypothetical population that is strongly suggested by intuition, the first inference is that the sampling technique (which includes laboratory procedures, *etc.*) is not statistically controlled and that greater pains must be taken, *e.g.*, in mixing solutions before withdrawing samples. Experience in biology and in industry over a period of nearly two

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[2] See Shewhart (S1, S2) and Simon (S3). Dr. Shewhart is the father of statistical control techniques in industry. The results achieved by Colonel Simon through the use of statistical control at the Picatinny Arsenal, Aberdeen Proving Grounds, have won him wide recognition and have been a major factor in convincing the Ordnance Department of the value of quality control.

decades shows that the foregoing is frequently the correct conclusion, and that a state of statistical control can be attained by persistent efforts directed toward improvement in technique. In some instances, however, the divergence is due to the choice of a hypothetical population which is unsuited to the phenomenon in question. For example, in studying the occurrence of larvae on a field, the distributions observed differ widely from those anticipated under the hypothesis that larvae are distributed on the field *independently* and *at random*. Biological considerations, *e.g.*, the fact that the eggs are laid in 'masses,' suggest that the presence of a larva in a given neighborhood increases the probability of there being others nearby. Accordingly, hypothetical 'contagious' distributions have been devised (69) with which the experimental facts seem to be in full agreement. In bacteriology such difficulties can usually be avoided by shaking suspensions well before taking counts, but as discussed in the following sections, other details of the technique may interfere with the realization of the hypothetical population.

PART I. DISTRIBUTIONS OF VARIABLES

A problem common to many branches of science is to determine whether values taken under one set of conditions differ significantly, in the *statistical* sense, from other values taken under other circumstances. The problem arises because individual values for any measurable quantity are rarely identical but show degrees of variation. Among the numerous causes may be cited: (a) errors in measurements because of lack of precision in the measuring instruments or ineptitude of the measurer; (b) variations among the individuals comprising the population—all men are not created equal. To decide if one set of data differs significantly from a second set, the statistician endeavors to define the characteristics of the populations from which the two sets were obtained and then to determine whether the two populations are identical in one or more respects. Our first problem, therefore, is to consider various types of populations and the methods by which the measurements of a variable can be used to calculate the significant parameters of each type.

THE BINOMIAL DISTRIBUTION

If the probability of an event occurring in *any* single trial is p, then the probabilities of it occurring exactly 0, 1, $\cdots$, x, $\cdots$, n times in n *independent* trials are given by the successive terms of the binomial expansion of $(q + p)^n$, where $q = 1 - p$ is the probability the event will not occur in any single trial.[3] The terms so generated form the *binomial distribution*, one of the most important hypothetical populations in biological research. It is sometimes called the *point binomial* since a variable so distributed can assume only integer values from 0 to n, and in consequence the probabilities are concentrated at these points. It is also referred to as the *Bernoulli Distribution* after its discoverer, James Bernoulli (1654–1705).

[3] For details see any text book on college algebra; particular the topics *Binomial theorem* and *Probability*.

A count, x, distributed in random samples in accordance with the above point binomial has a mean np and a standard deviation $\sqrt{npq}$, so that the observed proportion, $p' = x/n$, has a mean of p and a standard deviation $\sqrt{pq/n}$. When n is large, x is approximately normally distributed about its mean with the indicated standard deviation (23), as is p' also.[4] It is often convenient to employ $\theta = \arcsin \sqrt{p'}$ in statistical analyses instead of p', since for large values of n, the variance of θ is independent of the value of p, which is generally unknown. Tables are available (5, 6, 35) to facilitate this transformation. The point binomial possesses a reproductive property: the sum of N *independent* counts $x_1, x_2, \cdots, x_N$ based on samples of sizes $n_1, n_2, \cdots, n_N$ from the *same* population, i.e., p the same in each case, is binomially distributed with $n = n_1 + n_2 + n_j \cdots + n_N$. In consequence, a composite sample obtained by combining several independent samples may be regarded as a single large sample. The observed proportion in the composite sample, $\hat{p} = (x_1 + x_2 + \cdots + x_N)/n$, provides an unbiased estimate of p which contains all the information about p available in the data. For purposes of checking on *statistical control* it is advisable, however, to keep a record of the size (n_j) and count (x_j) for each of the respective samples.

Applications of the binomial distribution are numerous in genetics where Mendelian theory specifies the value of p. It is possible, however, to test whether a series of counts has properties characteristic of samples from a binomial distribution without knowing the value of p. Agreement with the binomial distribution is taken as evidence of the *independence* of whatever operations constitute 'trials' and of the *constancy* of p from trial to trial, which properties jointly comprise one form of *statistical control* often known as *simple sampling*.[5]

In sampling biological populations it is often desirable to test for agreement with the binomial to ascertain whether the sampling technique employed is statistically controlled. Likewise, when *random* samples are taken from each of several parts of a large body of material, or at different times from an ever-changing population, a test of whether the several samples may be regarded as samples from a *single* binomial, constitutes a test of whether p, the proportion possessing the characteristic under investigation, is the same throughout. If not, the population sampled is heterogeneous in respect to that characteristic, and heterogeneous material cannot, for purposes of inference, be treated statistically as though it comprised a single population.

Example: Table 1 (unpublished results of L. C. Ferguson and M. R. Irwin) gives data on the relative frequency of monocytes in the blood cells of a certain cow. Samples of 100 blood cells were counted at weekly intervals over a period of approximately two years; as is shown in the second column of the table, in the 113 samples, 19 contained exactly 4 monocytes, 2 contained exactly 12 monocytes, etc. Of the 11,300 cells counted 673 were monocytes, therefore $\hat{p} = 673/11,300 = 0.059558$. The expected frequencies, given by the successive

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[4] See the discussion of the normal distribution, p. 96 ff.

[5] For an excellent discussion of *simple sampling* and of the various types of departures from it, see Yule (123).

terms of

$$113 \, (0.940442 + 0.059558)^{100},$$

were computed with the aid of seven-place logarithms. A table of logarithms of $n!$ greatly facilitates the calculation, *e.g.*, table 49 of ref. (77). These calculated values are given in the third column of table 1; comparison with the observed frequency distribution indicates that the latter is more widely spread about the mean (5.9558) than would be expected in binomial sampling.[6] Various explanations of this apparent discrepancy are suggested: (a) the selection of the samples from the bloodstream was non-random; (b) the bloodstream was not homogeneous in the proportion of monocytes present, *i.e.*, the true proportion of monocytes varied from week to week; (c) the monocytes tended to occur in small clusters instead of being distributed at random within the bloodstream.

TABLE 1

Frequency of monocytes in blood of a cow

NUMBER OF MONOCYTES PER 100 BLOOD CELLS (X)	OBSERVED FREQUENCY F_X	EXPECTED FREQUENCY f'_X
0	0 ⎫	0.2 ⎫
1	3 ⎬ 8	1.5 ⎬ 6.5
2	5 ⎭	4.8 ⎭
3	13	10.0
4	19	15.3
5	13	18.7
6	15	18.7
7	12	15.9
8	10	11.7
9	11	7.6
10	7 ⎫	4.4 ⎫
11	3 ⎬ 12	2.3 ⎬ 8.6
12	2 ⎪	1.1 ⎪
Over 12	0 ⎭	0.8 ⎭
	113	113.0

It is not possible to infer from the data as arranged in table 1 which of these explanations is the proper one. From an examination of the original data sheets and from other evidence, however, it appears that (b) is the correct explanation.

THE POISSON SERIES

A distribution which is frequently of value in the description of biological material is the *Poisson Series*, the so-called law of small probabilities. Specifically, it defines phenomena whose occurrence is governed by the following

[6] A more precise method for comparing the two distributions is described in the section on *Testing for Agreement between Observed and Expected Frequencies.*

conditions: (a) the probability of occurrence, p, is very small, *i.e.*, order of 0.01 or less; (b) the number of individuals exposed to the 'risk' is extremely large so that the mean number of successes, or occurrences, np, is some small number; (c) the frequency of occurrence is represented by *small whole numbers*. Poisson showed that under these conditions, the probability of obtaining various frequencies is given by the series:

$$[1] \qquad e^{-m}, \ e^{-m}\frac{m}{1!}, \ e^{-m}\frac{m^2}{2!}, \ e^{-m}\frac{m^3}{3!}, \ \cdots \ \frac{e^{-m}m^x}{x!}, \ \cdots ,$$

$$\text{probability of} \qquad 0, \quad 1, \qquad 2, \qquad 3, \quad \cdots \quad x, \qquad \cdots$$

in which m is the average number of occurrences per sample.

A few properties of this distribution should be carefully noted:

(i) Its mean is m and its standard deviation is $\sqrt{m}$; hence an estimate of m, provides an estimate of its own error.

(ii) If in N independent samples from the same population an event occurs x_1, x_2, $\cdots$, x_N times respectively, then the observed mean $\bar{x} = (x_1 + x_2 + \cdots + x_N)/N$, provides an unbiased estimate of m, the expected frequency per sample, and furthermore, $\bar{x}$ contains all the information about m available in the data (30a, 34).

(iii) $\bar{x}$ is approximately normally distributed about m with standard deviation, $\sqrt{m/N}$, for any m if N is sufficiently large, and for any N (*e.g.*, for $N = 1$ so that $\bar{x} = x_1$) if m is sufficiently large. It is sometimes convenient to utilize the fact that the variable $y = \sqrt{\bar{x}}$ is approximately normally distributed about $\sqrt{m}$ with standard deviation $\sqrt{1/4N}$, which is independent of m, under these conditions (1, 2).

(iv) If T is the sum of N components which are independently distributed in a Poisson series of parameters m_1, m_2, $\cdots$, m_N respectively, then T itself is distributed in a Poisson series of parameter $m = m_1 + m_2 + \cdots + m_N$, so that T may be regarded as the frequency in a single sample from a Poisson series whose mean is estimated as T with estimated standard error $\sqrt{T}$.

(v) For small values of m the stability of the occurrence of events is very high—*e.g.*, when $m = 1$, the probability of no occurrence is $1/e = 0.368-$, which is also the probability of a single occurrence; the probability of 2 occurrences is $1/2e = 0.184-$, and the probability of more than 2 occurrences is therefore $1 - (5/2e) = 0.080$. There is considerable skewness with the 'tail' to the right; as m increases, this skewness diminishes although symmetry is attained only in the limit as $m \to \infty$.

It is of interest that the first experimental tests of the series were concerned with biological events. Bortkiewicz (8) showed that the number of men killed from the kicks of horses in each of 14 Prussian army corps for 20 successive years followed the Poisson law of small numbers. In 1907 "Student" (89), the famous chemist-statistician at a Dublin brewery, demonstrated that under somewhat idealized laboratory conditions the distribution of yeast cells on the squares of a hemocytometer conformed to a Poisson distribution; he also independently derived the law from considerations of how the yeast cells should distribute themselves in the squares of the counting chamber. Greenwood and White (44) investigated from the point of view of the Poisson distribution the ingestion of tubercle bacilli by phagocytes. Bortkiewicz's treatment contained the elements of statistical control of experiment, an application for which the series has been most useful. He discarded the records of 4 corps in which the

deaths were considerably higher than the others, allegedly, because the men in charge mistreated the animals so that they were more vicious than the horses in the other corps.

Following these publications an increasing number of diverse phenomena have been compared with the distributions predicted by Poisson series, including: emission of alpha particles from polonium; number of noxious weed seeds in a sample of timothy seed; number of umbrellas left on buses (statistical control, eliminate rainy days); death notices for men over 85 in the obituary column of London *Times*; wrong number connections in a telephone exchange, number of fires in New York City during a year (statistical control, eliminate July 4th and Election day); defects in a manufactured article; calls for a reference book in a University library. (See Thorndike (99) for an interesting discussion of many of these.)

Statistical control of bacterial counts by chamber method. Consideration of the conditions under which a count of yeast or bacteria is made in the various types of counting chambers leads to the conclusion that the distribution of organisms per square should follow a Poisson series since: (a) the probability that a given organism will be found in a given square is extremely small, but very large numbers of organisms are exposed to this small 'risk'; (b) the count per square will be some small whole number. "Student's" experiments with yeast were more for the purpose of verifying the law than for testing the methods of counting, but Wilson and Kullmann (114) definitely used the distribution for statistical control of a laboratory technique. They estimated numbers of the root nodule bacteria (*Rhizobium trifolii*) in a Petroff-Hausser counting chamber; because this organism produces gum, it clumps readily which frequently interferes with the reliability of results. Various refinements in technique were developed to overcome clumping, and the method as finally adopted was tested by counting the distribution of cells in the 400 squares of the chamber.

Figure 1 illustrates the results of four trials using the method of Thorndike (99) for testing agreement with the proper series. She has shown that if the relative frequency of obtaining at least c occurrences in data from a Poisson series is plotted on a special graph paper,[7] the points should follow a straight line drawn from the number at the base which corresponds to the mean number of occurrences (m). When the value of m is unknown—the usual case—the observed mean, $\bar{x} =$ (total number of organisms counted)/(number of squares examined) $= T/N$, may be taken as its estimate. The fit of the points to the theoretical lines in figure 1 is satisfactory in all four cases, especially in the center where the data are more reliable.

If the plotted points show a negative slope (*i.e.*, the points are to the left of the vertical in the upper portion and to the right of the vertical in the lower portion of the graph) the explanation of the non-conformance with Poisson

[7] Arithmetic probability paper. A scale proportioned to the normal probability curve is used for the ordinate, a linear scale for the abscissa. Logarithmic probability paper, with the abscissa scale in logarithmic units, permits the simultaneous portrayal of series with widely differing values of m.

sampling is often found in some restraint on large frequencies. Thorndike gives data on the number of calls in five-minute intervals from a pair of pay telephones which exhibit such a departure from Poisson expectations, "because of the fact that the number of calls which could possibly be made in five minutes from a group of two telephones is certainly finite and probably rather small." She gives also a sample of Perrin's data on particles in Brownian movement which show a similar departure and advances as an explanation that "it is
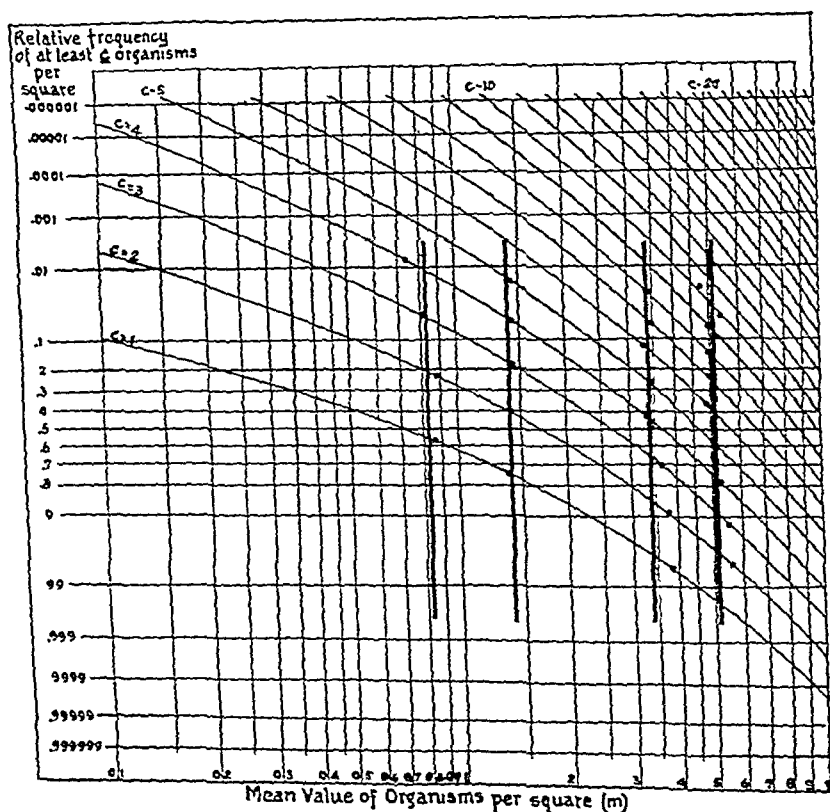
FIG. 1. COMPARISON OF OBSERVED DISTRIBUTIONS OF RHIZOBIUM TRIFOLII WITH THEORETICAL GIVEN BY POISSON'S EXPONENTIAL SUMMATION

The chart illustrates the use of a probability paper for testing whether data follow Poisson's law. A straight line is drawn from the point on the abscissa which corresponds to the mean number of bacteria per square; the experimental points represent the relative number of squares showing at least 1, 2, 3 . . . organisms. From Wilson and Kullman (114).

difficult to judge by the eye the number of particles visible simultaneously if that number is more than three or four." In bacterial counts such a departure from expectation on Poisson theory might arise from a tendency to underestimate the number of bacteria in crowded squares, or from a real restraint on large frequencies occasioned by competition among organisms. In either case greater dilution is a remedy. If the plotted points depart from the vertical with a positive slope, clumping or heterogeneity of material sampled (i.e., m not constant throughout) are generally the explanations, although such a

departure could arise from a tendency to overestimate the numbers of bacteria in crowded squares.

Like that of most graphical methods of analysis, the principal advantage of this method of testing conformance to Poisson sampling is its rapidity, and its chief defect is its failure to provide an objective criterion for judging whether the discrepancies observed are meaningful or merely fortuitous. Nevertheless, with experience it can become a valuable test of experimental technique, and, when used in conjunction with a method giving a probability measure of the discrepancies, it provides a convenient portrayal of the diagnosis.

TABLE 2

Comparison of the theoretical distribution with that observed when counting Rhizobium trifolii in Petroff-Hausser counter

Mean = 2.50

NUMBER PER SQUARE	THEORETICAL f_t	OBSERVED f_o	$f_o - f_t$	$\frac{(f_o - f_t)^2}{f_t}$
0	32.83	34	+1.17	0.04
1	82.08	68	−14.08	2.41
2	102.61	112	+9.39	0.86
3	85.51	94	+8.49	0.84
4	53.44	55	+1.56	0.05
5	26.72	21	−5.72	1.22
6	11.13	12	+0.87	0.07
>6	5.67	4	−1.67	0.49
Totals........	400	400		

$X^2 = 5.98$ $P = 0.43$ D.F. = 6

A more exact but slower method for testing the distribution is to compare the observed frequencies with the theoretical values obtained from the terms of the expansion

$$[2] \qquad Ne^{-m}\left(1 + \frac{m}{1} + \frac{m^2}{2!} + \frac{m^3}{3!} + \cdots + \frac{m^x}{x!} \cdots\right)$$

in which m is the true mean number of organisms per square, and N is the number of squares examined. When m is unknown, the observed mean number per square, $\bar{x}$, provides the appropriate estimate of m. Tables are available, which facilitate the determination of the theoretical frequencies, e.g., those of Soper (86), which provide the values of $e^{-m}m^x/x!$ to six decimals for $m = 0.1$ to $m = 15.0$. These tables have been reprinted by Pearson (Table 51, ref. 77). In the example given in table 2, a total of 1000 organisms were counted in the 400 squares, hence $\bar{x}$ equals 2.50. A comparison of the observed frequencies with those predicted by equation 2 ($m = 2.50$) is afforded by the first three columns of table 2, and indicates a reasonably close agreement; an exact evaluation of the agreement will be presented later in connection with the chi-square test of *goodness-of-fit*. The results of this and other similar trials indicate that

counts made with a Petroff-Hausser chamber under laboratory conditions follow the theoretical distribution when sufficient care is taken to break up clumps by thorough mixing of the suspension, thus confirming the reliability of the chamber count method when carefully executed.

How many observations should be taken? Property (iii) states that the mean number of organisms per square, $\bar{x}$, obtained from an examination of N squares has a standard deviation of $\sqrt{m/N}$, where m is the true mean number of organisms per square. The practical significance of this property is better appreciated when it is noted that the standard deviation of $\bar{x}$ is $100/\sqrt{Nm}$ per cent of the true mean, m, and is readily estimated as $100/\sqrt{T}$, from property (iv), T being the total number of organisms counted. Thus, for the data of table 2, $T = 1000$ gives an estimated standard deviation of 3.16 per cent for the mean (2.50) there shown. It follows, from property (iii), that the probability is approximately 0.95 that the mean observed here is precise to within 1.96 × 3.16% = 6.0 per cent, that is, the probability is approximately 0.95 that the interval 2.50 ± 0.15 includes the true mean number of organisms per square for the dilution here employed.[8] Alternatively, the precision wanted can be decided beforehand and sufficient squares examined to provide that degree of precision. Thus, if it is desired to have a probability of 0.95 that the observed mean will be within 10 per cent of the true mean, the total number of organisms counted will have to be at least 400 since 1.96 × $100/\sqrt{400}$ = 10. As it is the duty of every scientist to make the right kind of observations, it is also his duty to make a sufficient number. In the words of Shewhart (82)

"The applied scientist in order to be 'successful' cannot afford to make too many mistakes even though they be small, and in no case can he afford to make a mistake that is large enough to cause serious trouble. He does not consider his job simply that of doing the best he can with the available data; it is his job to *get enough data before making his estimates.*"

A modified method of counting. Counting of the 400 odd organisms necessary to give reasonable assurance that the observed mean number per square will be precise to within 10 per cent can be accomplished in practice by either: (a) employing a dilute suspension and examining a large number of squares, or (b) employing a dense suspension and examining only a few squares. Of these two alternatives the former is preferable for at least two reasons. First, with low cell concentration, a true Poisson distribution of the organisms is more likely to be realized—with heavy suspensions clumping, competition between the organisms, *etc.,* frequently distort their distribution, in consequence of which no confidence can be placed in the observed mean number of organisms per square as an estimate of the true concentration. Second, with high concentrations, mistakes in counting the organisms arise from difficulties in discerning the individuals and from mistaken estimates of their number.

To reduce mistakes in counting, Tippett (101) has proposed a modified method which may prove to be of considerable practical value in bacteriological work.

[8] See discussion of *confidence intervals* in Appendix.

It consists of recording as data merely the numbers of squares containing, $0, 1, 2, \cdots, t,$ and 'more than t' organisms, where t is some small number, say 3 or less. When $t = 0$, so that N_0 and N, the numbers of squares containing no organisms and the total number of squares examined, respectively, constitute the 'data', the maximum likelihood[9] estimate of m and its standard error are

$$\hat{m} = 2.303 \log (N/N_0) \text{ and } \sigma_{\hat{m}} = \sqrt{(e^m - 1)/N}.$$

Thus, for the data of table 2, $N_0 = 34$ and $N = 400$, giving $\hat{m} = 2.46$ with an estimated standard error of 6.65 per cent in contrast to the estimated standard error of 3.16 per cent corresponding to the complete enumeration of the 400 squares. Otherwise stated, when $m = 2.50$, a 'present-absent' enumeration of 400 squares is equivalent to a complete enumeration of 90 squares.

As one might expect, an optimum density exists for each value of t. For $t = 0$ ('present-absent' enumeration) this optimum is $m = 1.6$; the standard error of m from 400 squares in this case being 6.19 per cent in contrast to 3.95 per cent for a complete enumeration of all 400 squares. Alternatively stated, when $m = 1.6$ a complete enumeration of 160 squares is slightly less accurate than a 'present-absent' analysis of 400 squares. When t is greater than zero, the equations determining the maximum likelihood estimate, $\hat{m}$, of m cannot be solved directly, and solutions must be obtained by iteration. However, Tippett gives charts for $t = 1, 2,$ and 3 from which the value of $\hat{m}$ is readily obtained. He gives also a graph from which the standard error of $\hat{m}$ can be stimated. Thus, for $t = 3$, the relevant 'data' of table 2 are the total number of squares examined and the number with none, one, two and three organisms respectively. They yield $\hat{m} = 2.51$ with an estimated standard error of 3.35 per cent, which compares favorably with the result obtained by a complete enumeration of 400 squares, viz., 2.50 with an estimated standard error of 3.16 per cent. For $t = 1$ the optimum density happens to be $m = 2.5$ (from graph) and with $N = 400$ the standard error of the appropriate $\hat{m}$ is 4.3 per cent (from graph) so that when m is approximately 2.5 and a 'none—one—more-than-one' analysis of 400 squares is carried out, the probability is 0.95 that $\hat{m}$ is accurate to within 10 per cent, which is quite adequate for most purposes. Otherwise stated, a 'none—one—more-than-one' analysis of 400 squares is as accurate when $m = 2.5$ as a complete enumeration of 216 squares, and, without doubt, a great deal more rapid if Tippett's charts are at hand to facilitate the calculation of $\hat{m}$.

Statistical control of bacterial counts by plate method. Although many bacteriologists may never use the chamber method for counting organisms, the same can hardly be said about the plate method. Obviously then, of much more general application would be a procedure for statistical control of this technique. Since plate counts constitute samples from Poisson series, theoretically, the same type of test could be used as was described for the counting chamber, but considerations of time, labor, apparatus, and expense would render such a course *highly*

[9] An explicit account of the properties of maximum likelihood estimates is given in the Appendix. In this paper log refers to logarithms to base 10, ln, to base e.

impractical. If, however, as is often the case, series of counts are to be made on some material at certain intervals (daily, weekly, *etc.*) a statistical control on the precision of the plating technique is possible even though only 4 or 5 plates are used for each determination. Fisher (30, 34) has shown that if an index of dispersion,[10]

[3] $$D^2 = \frac{\Sigma(x_i - \bar{x})^2}{\bar{x}} = \frac{N\Sigma x_i^2 - (\Sigma x_i)^2}{\Sigma x_i}$$

(where Σ denotes summation over i from 1 to N) is calculated from the counts $x_1, x_2, \cdots, x_N$ provided by a set of N parallel plates, then in a sequence of such sets D^2 will be distributed according to the X^2 distribution for $N - 1$ degrees of freedom *when the plating technique is in statistical control*. The expression at the extreme right of equation 3 is generally the more convenient for purposes of calculation.

Many investigators have used this valuable contribution of Fisher's for checking the accuracy and reliability of plate counts made on various materials—often with surprising and revealing results. In the case of pure cultures grown on specially developed media by means of carefully standardized techniques, the observed distributions of D^2 have on the whole agreed quite satisfactorily with the theoretical. These studies include data of: 3-plate counts of *Escherichia coli* in milk (data of Breed and Stocking discussed by Fisher, Thornton and Mackenzie (34)); 3- and 4-plate counts of *Rhizobium trifolii* on yeast-extract agar (114); 4-plate counts of *Bacterium globiforme* and *Pseudomonas fluorescens* on nutrient agar (92). Using special selective media, a number of workers have shown that more heterogeneous populations likewise give a reasonable distribution of D^2 if the technique is carefully controlled; these populations include protein- and starch-splitting organisms and actinomyces in soil (52), and actinomyces and fungi in soil (54).

Whenever a very complex population such as that found in the soil is studied, however, departure of the observed distributions of D^2 from the theoretical distribution is almost always noted. The departure usually consists of a great excess of large values of D^2, but occasionally an excess of subnormal variation is also found. In either case the use of the data for drawing any profound conclusions is highly questionable. Instead, steps should be taken to locate the origin of the abnormal variation and, if possible, to eliminate it. In the studies to date this has not always been successful, but it has been of assistance on several occasions and has definitely led to the uncovering of unsuspected information in the data or of defects in the technique.

Using Cutler's data on the number of organisms found in daily counts of the

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[10] Fisher denotes this index of dispersion by X^2, and most writers have followed him in this usage, which has an excellent mathematical basis. We have made the change to D^2 in order to distinguish this index of dispersion from the X^2 *goodness of fit* criterion also discussed in the text (see p. 122 ff.). These two criteria are intimately related. It is our hope that, by using D^2 for the above and similar indexes of dispersion, and reserving X^2 for instances in which a frequency table (such as table 2) or a contingency table is concerned explicitly, the confusion which has arisen in some quarters may be lessened.

soil at the Rothamsted Experimental Station, Fisher, Thornton and Mackenzie (34) showed that in 156 sets of 4-plate counts and in 156 sets of 5-plate counts, an excess of both extremely low and extremely high values of D^2 occurred. If these were eliminated from the comparison, the remaining values agreed quite well with the theoretical. Searching for an explanation of the abnormal variances, they found that the excessively high values occurred in 'epidemics' during certain periods of the year. Although the origin of these epidemics could not be traced with certainty, evidences from other experiments suggested that they might be associated with the presence in the soil of certain species, usually of the spreading type, whose development inhibited the growth of other microörganisms. This not only led to an abnormally high variance (reflected in high values for D^2), but also seriously disturbed the reliability of the indicated mean.

The cause of the subnormal variance was even more obscure, but there was a suggestion that an apparently minor alteration in the preparation of the medium may have been a factor. These authors emphasize that an excess of low values for D^2 is just as much of a danger sign as excessively high values. Although no one is inclined to take too seriously results which show high variability, replicates in which the variation is abnormally low, far from exciting suspicion, are frequently exhibited as evidence of unusually reliable data. Fisher, et al. (34), cite, as an example, bacterial counts on cane sugar products in which the conditions which lead to the realization of the theoretical Poisson series were apparently operative in only about 45 per cent of the cases. An equal proportion was definitely subnormal with respect to variance, while 10 per cent were abnormally high. That some factor was concerned which disturbed random sampling was evident from the several sets in which the counts were practically identical on all six plates—a highly improbable result.

Harmsen and Verweel (52) likewise encountered an excess of high D^2 values from series of 10 plates used for counting bacteria in the soil of the Zuider Zee reclamation area in Holland. When soil or yeast-extract was added to the semisynthetic medium used, the excessive variability diminished but did not completely disappear.

Probably the most extensive and thorough exploration of methods for estimation of microörganisms in the soil by standard plating methods in which the D^2 criterion was used for statistical control is provided by the studies of James and Sutherland (54–57) at the University of Manitoba, Canada. As has been already mentioned, control experiments (4 plates) with P. fluorescens, B. globiforme as well as mixtures of these pure cultures plus sterile soil led to distributions of D^2 which agreed most satisfactorily with the theoretical values. An example from their studies is shown in figure 2; they concluded that their laboratory technique introduced no significant source of variation, and that difficulties with the counting must be ascribed to other factors. When the technique was used for counts on soil (493 sets of 4-plate data in 1937, 468 sets in 1938), an excess of high values of D^2 was obtained. Investigation revealed that time of plating after taking the sample definitely affected the variability encountered as is illustrated in figure 3. Seeking an explanation of this rather unusual source of variance, James and Sutherland investigated a large number
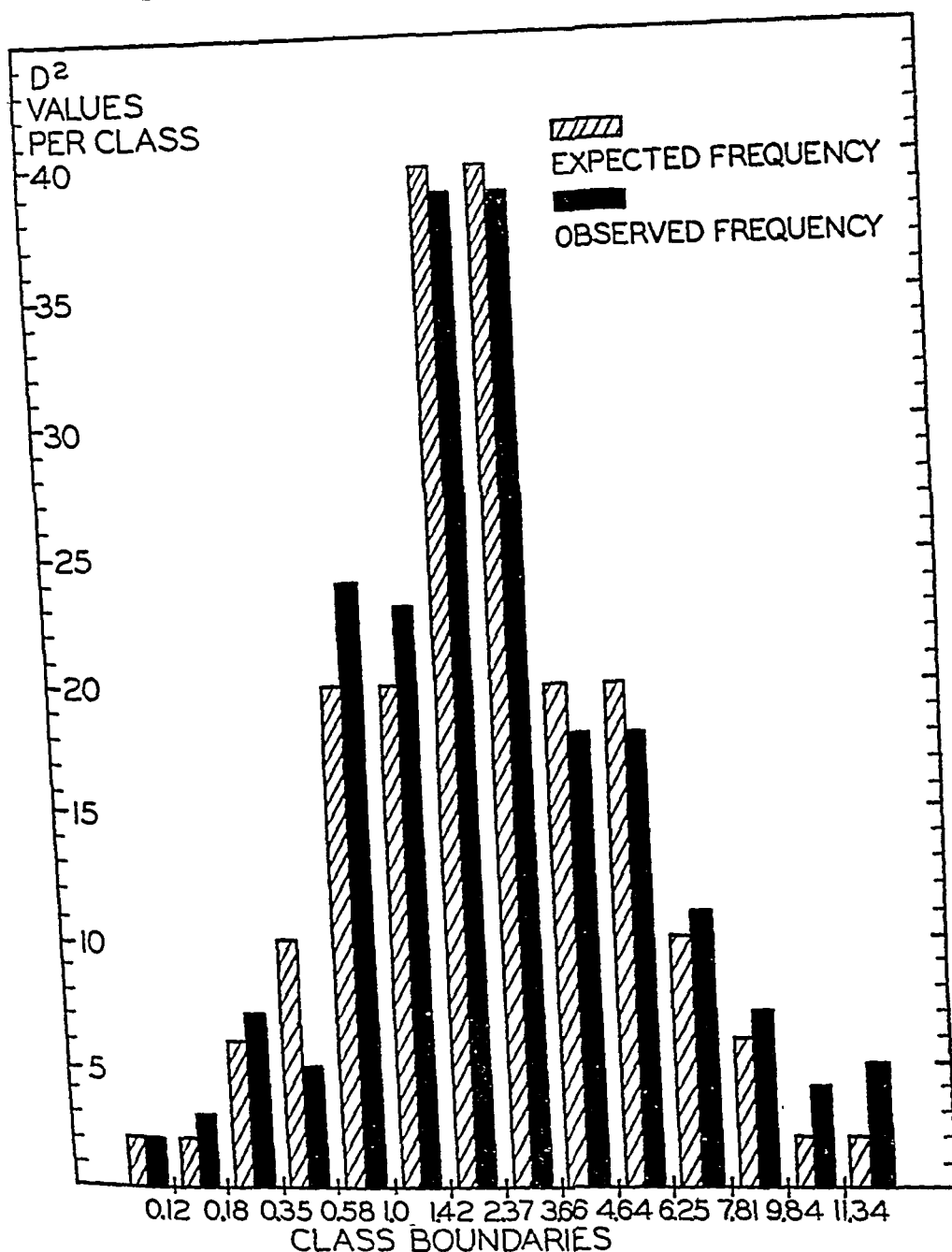
FIG. 2. HISTOGRAM OF D^2 DISTRIBUTION OF 200 SAMPLES OF PSEUDOMONAS FLUORESCENS PLUS STERILE SOIL

The value of D^2 for each set of 4 plates was calculated according to equation 3. The 200 values so obtained were classified according to the indicated class boundaries. The expected frequencies were calculated from an appropriate table of X^2 distribution with $n = 3$. Comparison of the *observed* with *expected* by the Chi Square goodness-of-fit test (see Part II) led to a value of $X^2 = 11.13$ corresponding to a probability of 0.6 that a worse fit might have arisen by chance. Both figures 2 and 3 are from the papers of James and Sutherland (54, 92). We thank these authors and the publishers of the *Canadian Journal of Research* for permission to reproduce these data.
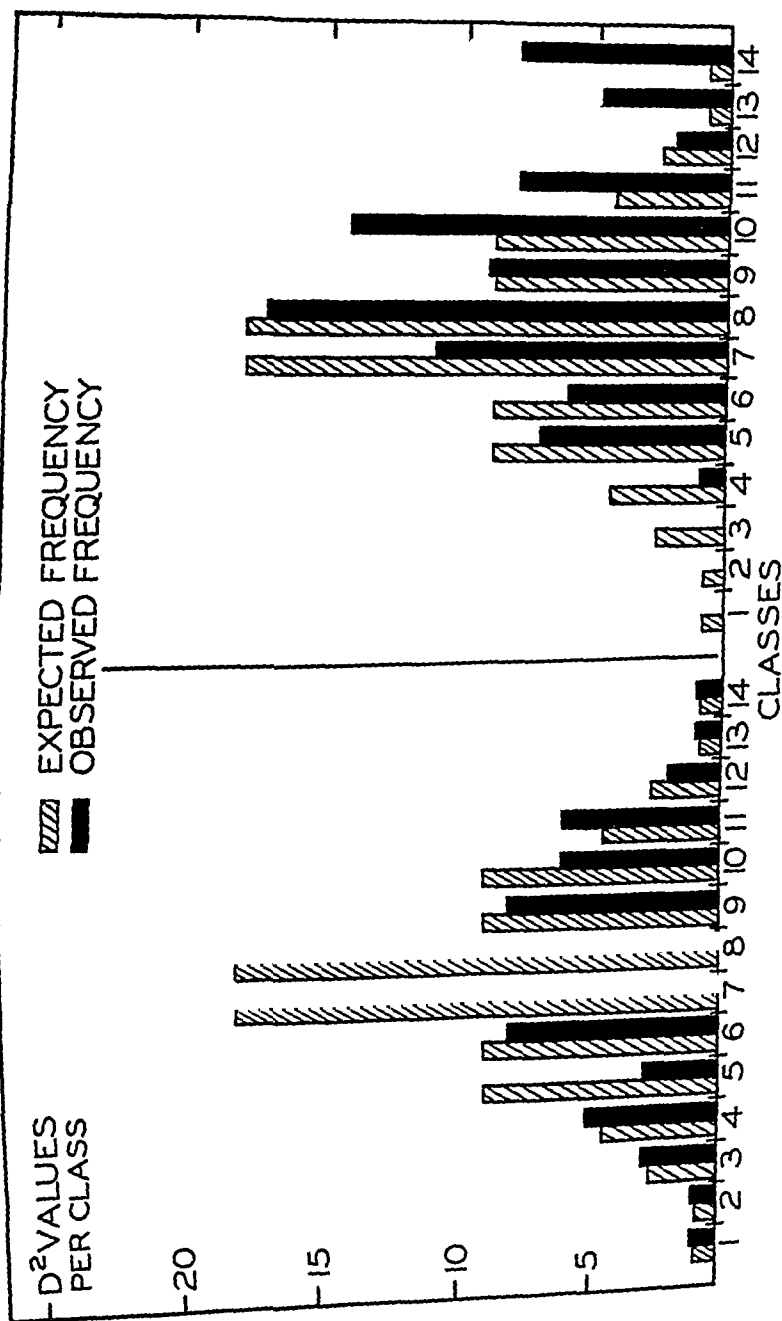
Fig. 3. Histogram of D^2 Values on Samples of Soil.

Left: Six replicates, 3 to 6 hours after sampling; $X^2 = 9.62$, $P = 0.73$. Right: four replicates, one day after sampling; $X^2 = 63.86$, $P < 0.01$. In this experiment further holding resulted in additional increases in X^2. Arbitrary numbers are used for class boundaries.

of possible factors and eliminated source and moisture content of the soil, technique, and medium. They did find, however, that associated with the abnormally large values of D^2 was the appearance on one or more plates of large numbers of pin-point colonies with or without large spreading colonies of the *Mucorales*. The presence of species of *Fusarium* or *Alternaria* as well as other fungi, which appeared only rarely, had no demonstrable effect on the value of D^2.

Control chart applied to plate counts. The foregoing method of appraising statistical control of plate counts from a sequence of sets of N parallel plates has three principal weaknesses. First, it cannot be applied until a large number of D^2 values has been obtained, by which time much of the data have become historic, and supplementary information which might throw light on the discrepancies is lost forever. Second, in forming a histogram (figures 2, 3) of the observed values of D^2 for comparison with the histogram expected on the supposition of statistical control, the order in which these values were obtained is disregarded, thereby discarding all characteristics of the *sequence* which are

TABLE 3

Probability levels of D^2[*]

P \ N	2	3	4	5	6	7	8	9	10
0.995	0.000	0.010	0.072	0.207	0.412	0.676	0.989	1.344	1.735
0.975	0.001	0.051	0.216	0.484	0.831	1.237	1.690	2.180	2.700
0.500	0.455	1.386	2.366	3.357	4.351	5.348	6.346	7.344	8.343
0.025	5.024	7.378	9.348	11.143	12.833	14.449	16.013	17.535	19.023
0.005	7.879	10.597	12.838	14.860	16.750	18.548	20.278	21.955	23.589

N stands for the number of plates in the set.

P denotes the probability of a value of D^2 exceeding the value given in the body of the table when a state of statistical control prevails.

[*] Taken from table calculated by Thompson (98).

intimately associated with order. Third, in a laboratory where replicate plate counts are made at regular (or irregular) intervals, it does not provide a basis for action (acceptance or rejection) with respect to current determinations. A statistical control technique which does not suffer from these weaknesses is the *control chart method* developed at the Bell Telephone Laboratories by Dr. Walter A. Shewhart and now employed in various industries and by the United States Army Ordnance Department.[11]

The application of the control chart procedure to a sequence of values of D^2 is simple because the distribution of D^2, when a state of statistical control prevails, depends only on the number of plates involved. Table 3 gives probability

[11] The best general introduction to the control chart method is provided by the *American War Standards* published and sold by the American Standards Association (29 West 39th Street, New York City):

Z1.1 (1941) *Guide for Quality Control*
Z1.2 (1941) *Control Chart Method of Analyzing Data*
Z1.3 (1942) *Control Chart Method of Controlling Quality During Production.*

levels useful in constructing control charts for D^2 values. Figure 4 shows a control chart for some of A. R. Colmer's plate counts of total bacteria in 1:50,000

FIG. 4. CONTROL CHART FOR PLATE COUNTS OF BACTERIA IN LAKE MENDOTA MUD
Upper and lower heavy lines represent control limits outside of which experimental points should fall no oftener than 1 in 40 on average. These limits change because the number of plates counted vary, since one or more of the samples plated at each date had to be discarded because of spreading colonies. The dotted line represents the value of D^2 for $P = 0.5$; hence one-half of points should be above, one-half below.

dilutions of Lake Mendota bottom mud. *When a state of statistical control prevails*, the upper heavy line is the control limit which should be exceeded by only 1 point in 40 on the average, and the lower heavy line is the control limit

below which only 1 point in 40 should fall on the average. The control limits wag up and down in a manner dependent only on the number of plates of colonies counted. This varies since, although 8 plates were always prepared, film and other factors caused the rejection in every instance of one or more plates before the actual counts were made. Three of the first four sets of plates represented here gave D^2 values above the upper control limit, indicating excessive variation between the counts on the parallel plates. Reference to notes made at the time of counting, revealed that *Bacillus mycoides* was recorded as a disturbing factor in the plates rejected before counting, and the plates retained may have been affected to some extent by this factor. It is concluded that the mean counts corresponding to these points are not trustworthy. The sixth point is just below the lower control limit. No remarks were on record in the laboratory notes which might explain this extremely close agreement among the plates, and it was decided to regard the result as fortuitous. Beginning with the fifth, the points indicate a state of statistical control. As a further check on control, a central line corresponding to $P = 0.50$ has been added to the chart, and it may be noted that, beginning with the fifth point, six points are above and four points below this line—an excellent agreement with expectation.

The above choice of control limits may be expected to lead us to look for trouble once in 20 times when statistical control prevails. Therefore, if in a long plating program, experience shows that roughly 1 point in 20 lies outside these control limits, *and* that the great majority of these are 'false alarms', i.e., no assignable cause for the discrepancy is discovered, then it will be desirable to use the upper and lower 0.005 limits so that 'false alarms' will arise only 1 time in 100 so long as statistical control prevails. Whatever limits are used, the median ($P = 0.50$) line should be drawn, and the occurrence of a statistically significant excess of points above (or below) this line, or long runs of points above (or below) this line, provides as much evidence of lack of statistical control as does the falling of points outside of the limits.[12]

The use of D^2 as a check on statistical control is not limited, of course, to plate counts. When direct counts by the microscopic or the chamber method are made in duplicate (or more), D^2 can be used to check the statistical control of the technique. When only two counts are involved,

[3a] $$D^2 = (x_1 - x_2)^2/(x_1 + x_2),$$

and the control technique is readily applied.

Dilution count

Estimation of organisms by noting growth in successive dilutions was introduced early in bacteriology, but except for the important test for *Escherichia coli* in water, milk, and other products, its potential usefulness has been appre-

[12] The expected number of runs of length r above the theoretical median line (or below the median line) in a succession of m points is $(m - r + 3)/2^{r+2}$ for $1 \leq r \leq m - 1$ and $1/2^r$ for $r = m$. If the runs of length r on both sides of the theoretical median line are counted, the expected number is twice that given above. See W. G. Cochran (20).

ciated only recently. The dilutions are usually made in units of 10, and for many years the interpretation was simple and erroneous. If growth was obtained in a dilution of 10^{-2} but not in 10^{-3}, the count was said to be somewhere between 100 and 1000, which was probably true. If, however, a skip occurred (e.g., growth in 10^{-2}, but not in 10^{-3}, growth in 10^{-4}, and no growth in the higher dilutions), it required an official ukase to obtain agreement. Officially the count was 1000 in this instance, since in case of skips the decree was that the result to be taken was the reciprocal of the dilution next higher than the smallest one giving a positive test. Although this solution may have worked satisfactorily in practice, it gave no greater assurance of accuracy than did the proposal to a state legislature to make the 'legal' value of π exactly 3.

The correct solution of the problem has occupied the statistician for many years, and judging by the recent output his interest remains undiminished. It is debatable whether bacteriologists have shared this concern, undoubtedly because few use the dilution method. Many may even question the appropriateness of including in this review an analysis of the rather extensive literature on this subject, arguing that the problem has intrigued the mathematician out of proportion to its practical value to the bacteriologist. Ample justification for doing so, however, exists. First, although no other aspect of bacteriology has been so thoroughly examined from the point of view of statistics, many important contributions have been published in journals seldom consulted by workers in this field. Second, important decisions affecting the health of all citizens are made in sanitary water analysis based on results of the test; correct interpretation of these is essential. Moreover, extension of the method to enumeration of organisms other than *E. coli* may provide a useful tool hitherto neglected. It appears to be superior to the plate count in certain cases of mixed populations for which selective media are used and may also prove useful in estimating organisms in unusual types of industrial products, e.g., pickle brine in packing houses (124). Finally, it is emphasized that, although the principles involved have been developed from the point of view of estimating numbers of viable bacteria, extension of the reasoning and mathematics to other problems in bacteriology is possible, for example, number of bacteriophage particles in a suspension (17), direct count on bacterial smears (103), infestation of an animal by insects (48), and securing a pure culture of an organism by dilution (30). Because of these and other possible applications of the general theory on which the dilution count is based, the statistical literature on the method will be examined critically and in some detail.

Underlying probability theory. Before a satisfactory evaluation of the statisticians' contribution to this problem is possible, we should consider briefly what might be termed the philosophy of the various attempts to answer the following type question. Given a certain result, what can we say about its 'cause'? For example, if growth is obtained in a number of tubes of a medium when inoculated with a known dilution of bacteria and no growth in others, what is the 'best' estimate of the number of organisms in the original suspension?

First, let us consider the inverse probability approach. In calculating the 'most probable density' which gave rise to an observed result and its probability limits, it is assumed

that the sample is from a long (strictly infinite) sequence of samples in which all densities within certain limits occur with definite relative frequency. Suppose we obtain the result.[13] '5/10 in 1 cc'. From the appropriate equation to be developed in the next section, the relative frequency with which each density per 100 cc, x, will 'produce' the result '5/10 in 1 cc' may be calculated, and using these values as the ordinates corresponding to the proper x, a graph such as is shown in figure 5 is constructed. The curves shown in this figure correspond to the case where all admissible bacterial densities are *a priori* equally probable. Then, if in a given type of research, all densities within some range do occur with equal relative frequency in the long run, it follows from Bayes' Theorem[14] that when '5/10 in 1 cc' is observed it will have resulted from $x = 69$ in more cases in the long run than for any other single value of x. Therefore, if under these circumstances it is stated that $x = 69$ whenever '5/10 in 1 cc' is observed, this statement will be correct more frequently in the long run than if it were stated that x was some other number, such as 50, and it is in this sense that 69 is the 'most probable value' of x. Likewise under these circumstances it can be shown that 99 per cent of the results '5/10 in 1 cc' will be 'produced' in the long run by values of x less than 189, and it is in this sense that in a single such instance it is permissible to say that the probability is 0.99 that x is less than 189.

Unfortunately this correspondence with long-run experience in a sequence of actual assays depends upon the validity of assuming that in the long run all admissible values of x will occur with equal relative frequency. If certain values of x occur more frequently than others, so that the *a priori* distribution of x is not a constant within some range of x and zero elsewhere, then the 'most probable value' and the 'probability limits' for x will differ in general from those found by the above procedure, but can be found with the aid of Bayes' Theorem *when the a priori distribution of x is known*. When the *a priori* distribution is unknown, then information essential for the application of Bayes' Theorem is lacking.

Lack of factual information regarding the range of x, and of the relative frequencies with which values of x occur in a particular kind of research certainly do not constitute sufficient reason for assuming that all values of x within some range occur with equal frequencies. It is the merit of Bayes' Theorem, not its weakness, that the inherent probabilities of the admissible values of an unknown quantity are taken into account, and, when from experience the *a priori* probabilities are known to a fair degree of approximation, better estimates can be obtained by utilizing this information than by ignoring it. "Bayes' Theorem is just as sound logically as any other part of the Theory of Probability, and may be trusted to give reliable results *when we can get a grip on it*. The trouble is that we so seldom can" (36, p. 128).

Until quite recently, in cases where the *a priori* information needed for the application of Bayes' Theorem was lacking, there appeared to be no alternative other than assuming such *a priori* distributions as seemed reasonable or convenient, and then proceeding with Bayes' Theorem undaunted. Two papers by R. A. Fisher (28, 30a) give impetus to a new way of looking at the problem of estimation. In these papers Fisher showed that, in repeated sampling from the same population, *maximum likelihood estimates* (see Appendix) based on a large number of observations would hover at least as closely about the true value of a parameter as estimates obtained by any other procedure from the same number of observations, and that in many instances this property extended to maximum likelihood estimates based on only a few observations. The search for 'most probable values' of a parameter was abandoned, therefore, and maximum likelihood estimates accepted as 'good' estimates, since maximum likelihood estimates would *generally* be 'close' to the true

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[13] '5/10 in 1 cc' means that from 100 cc of the suspension under investigation 10 subsamples of 1 cc were used to inoculate 10 tubes of which 5 showed growth. Since cc was used in the original publications, we use this symbol in these sections instead of the preferred ml used in the remainder of the paper.

[14] This important theorem of probabilty is concerned with the probability of causes. An excellent discussion of it is given by Fry (36, chap. V, sec. 95).

value of the parameter, *i.e.*, they will be 'close' to the true value except when the observations comprise an unusual sample from the population in question. Table 4 shows how this principle works and throws some light on the meaning of 'close' in the *above context*. The first column shows the possible outcomes with 10 tubes, the second gives the maximum likelihood estimates of x corresponding to these outcomes, and the third and fourth give the percentage of cases in which these outcomes (and hence these estimates) will be obtained when $x = 43$ and when $x = 138$, respectively. Thus, when $x = 43$, in about 50 per cent of the cases the result 3/10 or the result 4/10 will occur, leading x to be estimated as 36 or 51 respectively; only infrequently would an estimate of 0 or 10 be chosen; rarely would an estimate of 160 or more arise. Similarly, when $x = 138$, either 120 or 160 would be chosen about 53 per cent of the time, and an estimate of 36 or less would practically never be chosen. It should be noted, furthermore, that while the maximum likelihood estimate will generally be 'close', it cannot hit the nail on the head except in those cases where x happens to be one of the numbers which is a maximum likelihood estimate corresponding to a possible outcome of the experiment.

TABLE 4

Maximum likelihood estimates of, and upper 0.99 confidence limits for, bacterial density per 100 cc corresponding to all possible results 'in 1 cc,' and relative frequencies of occurrence when density is 43 and 138

RESULT IN '1 cc'	MAXIMUM LIKELIHOOD ESTIMATE	PERCENT OF CASES WHEN $x = 43$	PER CENT OF CASES WHEN $x = 138$	0.99 UPPER CONFIDENCE LIMIT
0/10	0	1.35	0.00	47
1/10	10	7.25	0.00	70
2/10	22	17.56	0.04	94
3/10	36	25.22	0.31	121
4/10	51	23.77	1.62	152
5/10	69	15.36	5.84	189
6/10	91	6.89	14.60	237
7/10	120	2.12	25.03	305
8/10	160	0.43	28.16	412
9/10	229	0.04	18.77	687
10/10	∞	0.01	5.63	∞

The final breaking away from the shackles of Bayes' Theorem took place about 1930 with the development of the concepts of *fiducial limits* by R. A. Fisher and of *confidence intervals* by J. Neyman (see Appendix). *The construction for the result '5/10 in 1 cc' of a confidence interval of the form $x < M$ corresponding to a confidence coefficient of 0.99 will illustrate the procedure:* Using the terminology customary in connection with tests of significance, an observed proportion, r'/n, will be 'significantly *less*' than a theoretical proportion, p, at the 0.01 level of significance if $\mathrm{P}\{r/n \leq r'/n \mid p\} \leq 0.01$, where $\mathrm{P}\{r/n \leq r'/n \mid p\}$ denotes the probability of observing a proportion as small as or smaller than r'/n when the true proportion is p. Now

$$[4] \qquad \mathrm{P}\{r/n \leq r'/n \mid p\} = \sum_{r=0}^{r'} \frac{n!}{r!(n-r)!} p^r (1-p)^{n-r}$$

and from tables of this summation (12, 24) it is found that $\mathrm{P}\{r/10 \leq 5/10 \mid p\} \leq 0.01$ for $p \geq 0.849$, so that 5/10 is significantly *less* than any proportion ≥ 0.849 at the 0.01 level of significance. It can be further shown that $p \geq 0.849$ implies $x \geq 189$ (63); hence $x < 189$ is the desired '0.99 confidence limit'.[15]

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[15] The value of x corresponding to a probability of 0.99 determined from the '5/10 in 1 cc' curve of figure 5 (see section on *Accuracy of Estimate*) also is 189. In this particular case

In the last column of table 4, '0.99 upper confidence limits' are given for x corresponding to each possible outcome 'in 1 cc'. The 0.99 confidence property of these intervals can be seen as follows. Suppose repeated sampling is being done from a supply for which $x = 13S$, then a *false* statement about x will be made *whenever* 0/10, 1/10, 2/10, or 3/10 occurs, since in these instances the inferences made will be $x < 47$, $x < 70$, $x < 94$, and $x < 121$, respectively. In these cases only will a false statement about x be made. But the probability of some one of these events occuring when $x = 13S$ is only 0.0004 (by adding the probabilities of these events), so that the probability of some one of the other events (*i.e.*, 4/10 through 10/10), each of which lead to a correct statement, is 0.9996. Therefore, if whatever ratio arises, the corresponding interval is used, then the probability of a correct statement is ≥ 0.99 when $x = 13S$. By virtue of the way in which these intervals were constructed they will have this same property for any value of x.[16]

The 'Best' Estimate. So far as the authors have been able to determine, McCrady (63) first approached the problem of estimating bacterial concentrations from dilution data with the aid of the theory of probability. He considered the selection of a single value to use as *the* estimate and the equally important question of the accuracy of the estimation. Acknowledgment was made of mathematical assistance received from Wm. D. Cairns, at that time Associate Professor of Mathematics at Oberlin College. Four cases were considered: (a) one dilution, one tube; (b) one dilution, several tubes; (c) several dilutions, one tube at each; and (d) several dilutions, several tubes at each dilution. No attempt was made in case (a) to obtain a single estimate of the number of bacteria. In cases (b) to (d), McCrady selected as the 'most probable number' the number which assigns the greatest probability to the event actually observed. Thus, the events '5/10 in 1 cc', '4/5 in 1 cc' and '9/10 in 1 cc' lead to the estimates 69, 160, and 229 bacteria per 100 cc, respectively, and not to 50, 80, and

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the values of x corresponding to a given probability level are identical, independent of whether inverse probability or maximum likelihood statistics are used. This is largely a coincidence and in general does not obtain. It should be noted that no claim is made in confidence interval theory that a *single* 0.99 confidence interval such as $x < 189$ will include the true value in 99 per cent of the cases in which it is employed, *i.e.*, in which '5/10 in 1 cc' occurs. The confidence coefficient 0.99 applies to the *entire set* of confidence intervals which as an aggregate constitute an estimation procedure. If, as the different possible events occur, the *corresponding* intervals are employed, then in the long run 99 percent of the inferences regarding x made with this set of intervals can be expected to be correct irrespective of whether x varies from case to case or remains the same. This distinction between the two forms of inference does not appear to be adequately appreciated among research workers in spite of the fact that Fisher, Neyman and others have stressed it for over a decade. By a coincidence, 0.99 confidence limits for x, determined from the outcome of several tubes at a single dilution, are identical with 0.99 probability limits for x based on the assumption that all admissible values of x are *a priori* equally probable. Similarly, for other levels of confidence. Therefore, it is true that, *if all values of x do occur with equal relative frequency*, a single 0.99 confidence interval such as $x < 189$ may be expected to include the true value in 99 per cent of the cases in which it is employed; if the relative frequency of the values of x is otherwise, this expectation does not obtain.

[16] Owing to the discontinuous nature of the variable r/n it is not possible to construct intervals such that the probability is exactly 0.99 that a correct statement will be made. This can be done when the variable observed varies continuously. Note that a correct inference will *always* result in the present situation when $x < 47$.

90 per 100 cc as might be inferred. As developed by McCrady from the inverse probability point of view, his 'most probable number' depends on the validity of regarding all admissible concentrations of bacteria as being equally probable before the event. The 'most probable numbers' turn out to be 'maximum likelihood estimates' as well, but as pointed out in the preceding section, from the point of view of maximum likelihood the justification of the choice does not depend in any way upon the relative frequencies with which admissible values of the unknown quantity occur. Such estimates are not regarded as 'most probable values' but are chosen on the basis of the manner in which they are distributed about the true value in repeated trials.

It will be instructive to consider in detail an example of McCrady's estimation process. Case (c)—one dilution, several tubes—lends itself especially well to such consideration. He assumes that the x bacteria in the V units of volume comprising the sample under investigation are distributed randomly and independently throughout this sample. It follows that the probability of a single unit of volume containing no bacteria is $[(V-1)/V]^x$. Thus, when V is 100 cc and 1 cc is taken, the probability of no bacteria in the 1 cc is $(0.99)^x$, and the probability of some (i.e., at least one) bacteria is $1 - (.99)^x$. If n samples of V volume units each were drawn at random from the solution under investigation and a subsample of 1 volume unit taken from each, then the probability of exactly r of the subsamples containing bacteria is

[5]
$$\frac{n!}{r!(n-r)!}\left[1-\left(\frac{V-1}{V}\right)^x\right]^r\left[\left(\frac{V-1}{V}\right)^x\right]^{n-r}$$

McCrady regarded equation 5, written in slightly different notation, as a sufficiently close approximation to the probability that exactly r out of n subsamples will contain bacteria when all n subsamples are taken from the same sample of V volume units. This is not strictly true since the reduction in number of bacteria is not necessarily proportional to the reduction in volume of fluid arising from withdrawal of the successive subsamples. As McCrady (63) notes, however, so long as n is small compared with V, the discrepancy will not be great. In figure 5, graphs of equation 5 for $V = 100$ are given when $n = 2$ and $r = 1$, i.e., for '1/2 in 1 cc', and when $n = 10$ and $r = 5$, i.e., for '5/10 in 1 cc', with x as abscissa and the probability of the event concerned as ordinate. Both curves attain their maximum values for $x = 69$, which is McCrady's estimate of the number of bacteria per 100 cc in either case. The maximum of equation 5 in general occurs at the value of x which is the solution of the equation

[6]
$$1-\left(\frac{V-1}{V}\right)^x \approx \frac{r}{n}$$

that is, by

[7]
$$x = \left(\log\frac{n-r}{n}\right)\Big/\left(\log\frac{V-1}{V}\right)$$

McCrady gives equation 6 in slightly different notation, but not 7, and notes that at a given dilution the results r_1/n_1 and r_2/n_2 yield the same x whenever these fractions are equal. The estimations will not be of equal accuracy, however.

While McCrady's 1915 paper might be said to have 'completely solved' the case of one or more tubes at a single dilution, as much cannot be said of his treatment of the case of one or more tubes at each of several dilutions. He

showed how to develop equations from which can be calculated the 'most probable number' of bacteria per cc corresponding to the various possible outcomes of inoculating several tubes at each of several dilutions *when all admissible numbers of bacteria per cc occur with equal frequency in the long run*. The equation being somewhat difficult to solve, he gave a table of the 'most probable numbers' for "most of the practically possible" results which may occur from the systems: (a) Two tubes 'at 10 cc', ten tubes 'at 1 cc', and (b) two tubes 'at 10 cc', ten tubes 'at 1 cc', and ten tubes 'at 0.1 cc'. In a subsequent table (64) he gave the 'most probable numbers' corresponding to all possible combinations for several special cases including those where 5 and 10 tubes are used at each dilution. As already noted, *these* 'most probable numbers' are also the corresponding maximum likelihood estimates. Wolman and Weaver (119), by making a few minor approximations, rendered McCrady's equations easier to solve, but their contribution lost its importance once tables of the solutions were available. Continuing with the type of reasoning he employed in the case of one or more tubes at a single dilution, McCrady indicated how, with the aid of Bayes' Theorem, probability limits for the number of bacteria per cc corresponding to the various outcomes of several tubes at each of several dilutions might be obtained. He did not attempt to derive any formulae, however, and thus left unsolved the matter of accuracy of the estimates he tabulated.

Others were studying the interpretation of dilution data at about the same time as McCrady, and shortly after the publication of his first paper these researches began to appear in print. W. F. Wells, in a series of papers (106–109) and in a joint paper with P. V. Wells (110), considered various ways of handling and interpreting dilution data. Objections to these methods have been raised by various writers, among them Cairns (15), who, as noted above, assisted McCrady with the mathematical portions of his analysis.

For the case of several tubes at a single dilution, Stein (87) proposed the use of what amounts to the maximum likelihood estimates of the bacterial density (*e.g.*, number of organisms per cc) from the observed proportion of *negative* tubes. A table of these estimates is given but the values shown are not accurate. Using the formula for the standard deviation of an observed proportion under simple sampling,[17] he presented in tabular form calculations of the number of tubes necessary to make the standard deviation of the estimated bacterial density equal to 10 per cent or to 5 per cent of the true bacterial density. He found that, for bacterial densities between 1.058 and 1.900 per cc, the number of tubes needed to reduce the standard deviation of estimated density to 10 per cent of the true values is between 155 and 165, and that outside of this density range the number of tubes needed mounts rapidly. Fisher (30) has given the minimum number as "about 155" at a density of 1.6. In a second paper, Stein (88) furnishes a chart from which the estimated density corresponding to an observed proportion of positive tubes can be read. The mathe-

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[17] Stein refers to the *standard deviation* as the "mean error", "expected error", and "probable error". These latter expressions generally have a different meaning in statistical papers.

matical discussion here is somewhat fuller, and two different approaches are given.[18]

Apparently unaware of McCrady's work, Greenwood and Yule (45) approached the problem of interpreting dilution data essentially as McCrady had. They introduced one important simplification (employed independently by Stein), which has been utilized by most subsequent writers. Instead of considering the preparation of the tubes at one or more dilutions as *subsampling* from a sample of volume V, which McCrady takes to be 100 cc, and attempting to estimate the number of bacteria x, in *this* sample, Greenwood and Yule regard the preparation of the tubes as constituting sampling from the supply itself, which is considered as having a practically infinite volume and in which the density of bacteria per cc is λ. In this way, the difficulties arising from changes in the volume as successive tubes are prepared is avoided, and the formulae are also somewhat simpler.[19] A table is given of the maximum likelihood estimates—interpreted as 'most probable values' as in McCrady—for all results involving at least one positive and at least one negative tube, corresponding to the use of 10 or less tubes at a single dilution. Where comparisons are possible, these estimates of Greenwood and Yule agree with Fisher's values (30) except for occasional difference of unity in the last digit.

On the general question of what series of dilutions to use and with what numbers of tubes at each dilution, Greenwood and Yule remark: "One obvious condition, strangely overlooked, is that the size of any one sample should be greater than the sum of the sizes of the smaller samples. Otherwise the observer is simply asking for 'inconsistencies' in his results.[20] A geometrical series fulfills the required condition ... [and] ... seems also a natural one to use as the chance of an inconsistence is the same at every point of the series [when an equal number of tubes is used at each dilution]. ...[With a single tube at each dilution] r being the (ascending) ratio of the series, the chance of an inconsistence between any adjacent pair of samples ... is $1/(r + 1)$."

Basing his analysis on the results of Greenwood and Yule (45), *which were* obtained with the aid of Bayes' Theorem and the assumption that all *admissible*

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[18] We have found two errors in connection with the second approach. *First, Stein's* relation between a, the number of bacteria per cc in the supply, and Q, the expected proportion of negatives in N tubes when n cc are introduced into each tube, is incorrect. The correct formula is $a = -(1/n) \ln Q$. Formula XI (87, p. 254), expressing the *relation in* terms of Q for the simple sampling deviation of the number of bacteria per cc in a random sample of Nn cc, is also incorrect because the *incorrect relation between a and Q* was used.

[19] Since the limit of $[(V - v)/V]^x$ is $e^{-r\lambda}$ as V and x both increase indefinitely with x/V tending to λ as a limit, the latter (with N for v) appears in the formulae of Greenwood and Yule where the *former* occurs in McCrady's formulae. Accordingly, by means of this relation, one can pass from McCrady's results in terms of V, x, and r to Greenwood and Yule's results in terms of λ and N, and *vice versa*.

[20] When a larger proportion of tubes give positive results at a certain dilution than at a *lesser* dilution, the result is said to be 'inconsistent' or 'anomalous'. Thus, with one tube at each of three increasing dilutions, the results $++-$ and $+--$ are 'consistent', whereas the results $+-+$ and $--+$ are 'inconsistent'. (In this quotation the expressions in square brackets have been inserted by the present writers.)

densities were equally probable *a priori*, Reed (78a) made a detailed study of the case where the estimation is based on a set of five tubes containing 100 cc, 10 cc, 1 cc, 0.1 cc, and 0.01 cc, respectively, of the solution under investigation. He notices that the most probable densities (which are maximum likelihood estimates also) corresponding to the various possible outcomes form a "yardstick" with "very coarse divisions," and for this reason this set-up "is suitable for grading waters that vary widely in the extent of pollution." He notes, and illustrates graphically, that for the so-called consistent outcomes, the estimated density and its accuracy (from the inverse probability viewpoint, at least) are almost entirely determined by the two tubes where the results change from + to −. In the so-called inconsistent cases, however, both changes of sign play a part in determining the estimated density and its estimated accuracy, and "it would be better to regard them as further subdivisions of the yardstick, having their own probabilities, than to treat them as inconsistencies." He states that the use of five tubes at a single dilution will be more accurate than five tubes in geometric dilution series, at least when accuracy is evaluated by the Bayes' Theorem approach, provided the five identical tubes are run at the most suitable dilution. Except for cases where the customary neighborhood of the bacterial density is known, the difficulty lies in picking the most suitable dilution beforehand.

Fisher (28) considered the case of several tubes at each of several dilutions from the viewpoint of the method of maximum likelihood, and gave in condensed notation the general equation determining the maximum likelihood estimate of the bacterial density. Except for notation, this equation is identical with that obtained by Greenwood and Yule for the 'most probable density' on the assumption that all admissible densities are equally probable *a priori*, and with the equation obtained by Halvorson and Ziegler (49) for the 'most probable value' under the same assumption. The last mentioned writers claim that their equation is more general than the equation of Greenwood and Yule. There appears little justification for this claim, as noted by Swaroop (93), who also shows how, by a slight rearrangement of the equation, its solution may be obtained more readily. Swaroop prepared a table of estimates for the case where the dilutions are in the ratio 1/2, 1/10, 1/100 with a single tube at the first dilution and 5 tubes at the other two dilutions, and for the case where the dilutions are in the ratio 1/10, 1/100, 1/1000 with 5 tubes used at each dilution. He remarks that two mistakes in McCrady's tables have been uncovered, and that McCrady's approximate values (when the estimates exceed 20) have been replaced by values correct in the units place. Halvorson and Ziegler (48, 49) have tabulated the estimates corresponding to code numbers[21] likely to be encountered in practice for the case where the (ascending) dilution factor is 10, and 10 tubes are used at each dilution.

[21] If 10 tubes are used at each of five consecutive dilutions, an overall code of 10-10-10-8-3 might be observed, meaning that all 10 tubes show growth at the first three dilutions, only 8 of the 10 in the next, and 3 of the 10 in the final. The critical code for entering the table is 10-8-3, and multiplication of the tabulated estimate by the appropriate power of 10 would give the density per cc, per 100 cc, *etc.* as desired.

As already stated, the 'most probable density' derived on the assumption that all admissible densities are equally probable *a priori* advocated by McCrady, Greenwood and Yule, Stein, Reed, Halvorson and Ziegler, and others is identical with the estimate indicated by the method of maximum likelihood advocated by Fisher and Swaroop. Therefore, so far as choice of a *single* estimate of λ is concerned, these writers are unanimous, although the reasons for their choice differ. Gordon (40–42) takes issue with all of the foregoing writers—although he singles out the work of Halvorson and Ziegler for specific criticism—and proposes an estimate, which, though based on Bayes' Theorem and the assumption that all admissible densities are equally probable *a priori*, differs from the 'most probable density' corresponding to this assumption. *If this assumption is valid and simple sampling prevails, then, in our opinion, a table of the 'most probable densities' being available, it is curious to advocate any other estimate, since the 'most probable density' can be expected under these conditions to hit the nail on the head more often than any other.* While Gordon does not question the use of Bayes' Theorem with uniform *a priori* distribution for λ, he does question the validity of the simple sampling assumption, which underlies the work of the aforementioned writers, that the individual bacteria are distributed in the fluid *independently at random*. It is his thesis that bacteria "exert a certain mutual uniformizing influence on one another" so that "the numbers of bacteria caught up in [a series of samples] are somewhat more closely clustered about the true average density, than we should compute them to be on the basis of the above assumption of complete randomness." Gordon apparently claims (41, p. 169) that his method is less affected by bias from this source, but it is difficult to see how he reaches this conclusion since his analysis utilizes a formula (equation 3, p. 170) which is based on simple sampling and which differs only in notation from the fundamental formulae employed by the other writers.

Pearson (74) has given a very careful evaluation of Gordon's paper, including a discussion of the relative merits of the inverse (Bayes' Theorem) and direct (maximum likelihood, confidence interval) methods of approach. Noting that the 0.95 confidence intervals are quite broad, (see section on *Remarks and Suggestions*, especially table 6), Pearson remarks that "having regard to this, the differences between the single value estimates are of little importance." In reply to Gordon's implications that the maximum likelihood estimates are biased, Pearson states "if a method of obtaining accurate fiducial limits were available, the bias in the single-valued estimate would be of no importance." An accurate chart of confidence intervals would provide such a method, and would also make possible a check on agreement of actual samples with expectancy under simple sampling, *i.e.*, a check on *statistical control* of laboratory technique.

Accuracy of the 'Best' Estimate. In the preceding section we have focused our attention primarily on the efforts of different contributors to determine the 'best' single value from a given set of laboratory results. Although knowledge of the 'most probable' number of organisms is of undeniable importance, it has little significance unless something about its accuracy is also known. *We shall*
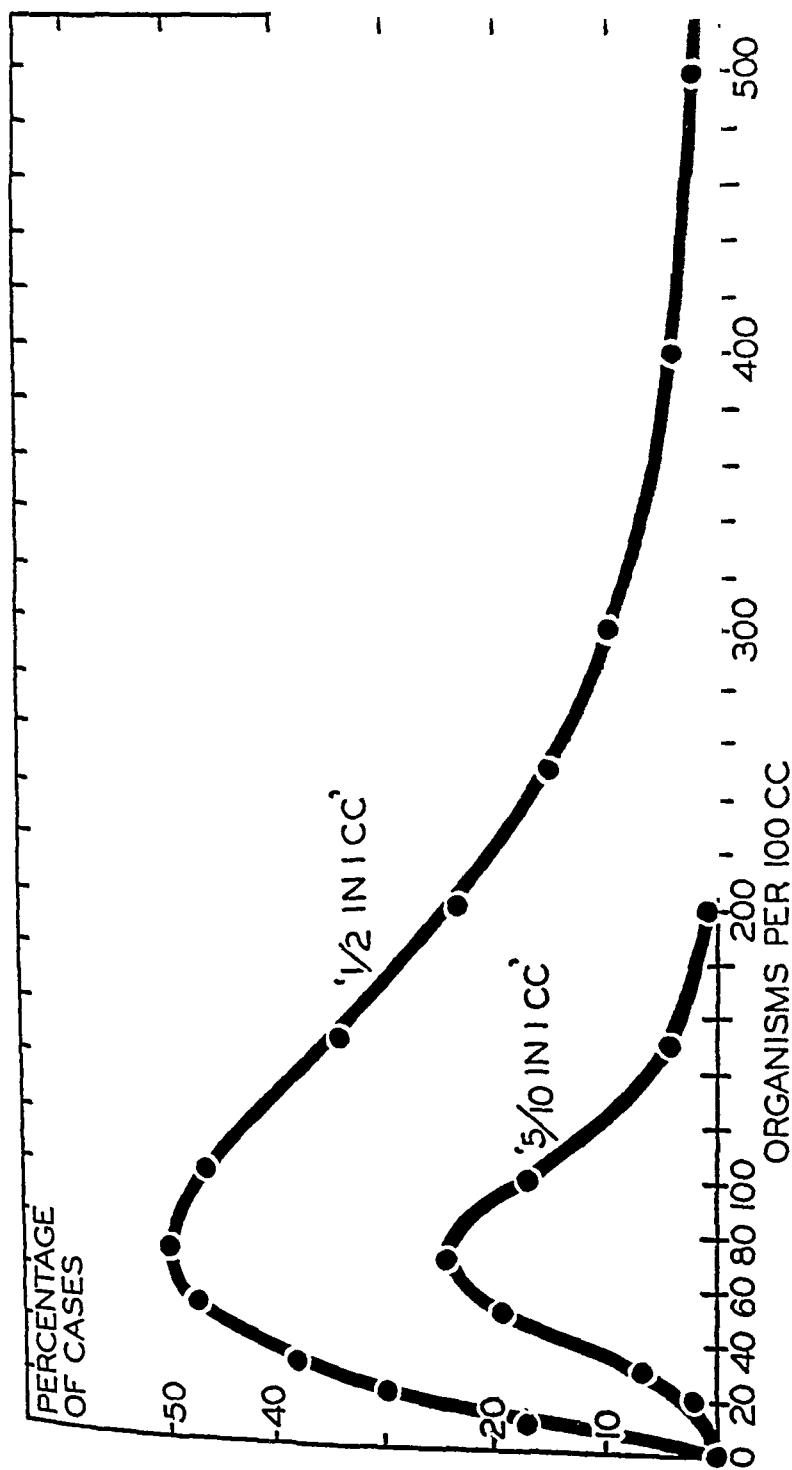
FIG. 5. EXPECTED FREQUENCIES OF TWO POSSIBLE RESULTS FOR VARIOUS TRUE DENSITIES OF BACTERIA

When the true number of organisms is that given in the abscissa, the observed result, 'one tube in two inoculated show growth' (upper curve), will on the average be observed in the indicated percentage of cases. The lower curve represents the result, 'five tubes of ten inoculated show growth.' Note that 'most probable number' (maximum in curve) is the same for both curves since in both instances 50% of tubes show growth. The 'range' (for example, the number of organisms which will include 95% of the cases) however, is much smaller with the larger number of tubes. Chart constructed after McCrady (63).

now consider how various workers have sought to determine this property of their estimates. McCrady's (63) approach to the problem of accuracy is best summarized in his comments regarding the curve for '5/10 in 1 cc' (figure 5): "Inspection of the corresponding curve ... shows that the practically possible numbers of bacteria have a range from about 15 to about 200 per 100 cc." He gives no range for the '1/2 in 1 cc' case but, presumably, he would consider the "practically possible" range from 1 to 600 per 100 cc. To explain his reasoning, he points out that by using Bayes' Theorem with equal *a priori* probabilities, "the ordinates of the curve give the relative probabilities that the corresponding abscissae were responsible for the result," so that "the general shape of the curve indicates roughly the degree of confidence which may be assigned the inclusion of x within certain limits." As an elaboration of this procedure, he notes that, by summing the ordinates of the curve from $x = 0$ to $x = k - 1$ and dividing by the sum of the ordinates from 0 to ∞, the probability that the samples contained less than k bacteria per 100 cc can be obtained. Formulae are given (63, p. 197) to facilitate these summations. As an example he shows that, all admissible values of x being assumed equally probable *a priori*, the occurrence of the result '1/2 in 1 cc' implies that the probability is $89.937/99.503 = 0.90386$ that x is less than 300. With the same assumption, it may be shown that the results '1/2 in 1 cc' and '5/10 in 1 cc' imply that the probability is .99 that x is less than 527 and less than 189, respectively. Since both of these results yield 69 as the 'most probable value', the greater accuracy of the result corresponding to 10 tubes is evident.

In his 1919 paper, Stein (87) furnishes a curve showing the estimated density $\pm$ its standard deviation for 30, 100 and 360 tubes. He appears to have used the standard deviation of a_N, the number of bacteria per cc in a *random* sample of Nn cc from a supply in which the actual density is a per cc. Since a_N cannot be observed, its standard deviation is not of great practical value. What can be observed is the proportion, say q, of negative tubes of N inoculated with n cc of the supply; from q an estimate, say $\hat{a}$, of a can be calculated: $\hat{a} = -(1/n) \ln q$. To a first approximation, the standard deviation of $\hat{a}$ is $\sqrt{(e^{na} - 1)/N}$, of which the estimate from the data is $\sqrt{p/qN}$, where $p = 1 - q$ is the observed proportion of positive tubes. This latter formula was given by Greenwood and Yule in different notation. The standard deviation of $\hat{a}$ will always be larger than that of a_N since $\hat{a}$ depends merely on the presence or absence of bacteria in the Nn cc withdrawn and takes no account of the number of bacteria in the positive tubes. Thus for $\hat{a} = 0.7$, corresponding to $q = 0.50$, the correct limits are about 20 per cent wider than indicated in Stein's curves. However this does alter the fact, noted by Stein, that above densities of about 1.9 per cc (expected proportion of positive tubes, above 0.85), these curves become so flat that small changes in the observed proportion cause large changes in the estimated density.

To obtain limits which are today interpreted as confidence limits, Stein proposed the use of Tschebycheff's inequality—in the first paper he appears to suggest that this be applied with the standard error of the observed proportion of positive (or negative) tubes to obtain limits for the expected proportion, from

which limits those for the bacterial density could then be calculated from the equation relating these two quantities. In the second paper he inadvertently drops the inequality sign (and the inequality is generally great) and apparently proposes its use with the standard error of the estimated density to get limits for the actual density. These two procedures are not equivalent; thus, with 100 tubes and an observed proportion close to 0.5, the former would assign a confidence coefficient of *at least* 0.96 to the density range of 0.29 to 1.40 bacteria per cc, and the latter to a range of 0.35 to 1.05 bacteria per cc. Actually the limits obtained by either of these processes are too wide, since Tschebycheff's inequality is extremely loose—a correct range for the above confidence coefficient would be approximately 0.51 to 0.92 bacteria per cc.

Fisher (28), employing a method available only for maximum likelihood estimates, obtained the variance of ln λ, where λ (which he denotes by n) is the maximum likelihood estimate of the density, for the case where s, the number of tubes used at each dilution is large. When s is small, Fisher's expression will give the variance of ln λ to a first approximation. Only minor alterations are necessary to extend Fisher's expression to cover the case where unequal numbers of tubes are used at the respective dilutions, and, although Fisher's derivation is for the case where the dilutions are in geometric progression, the validity of his result can be extended without difficulty to cover other arrangements.

Using Fisher's method and notation, Swaroop (93) derived an expression for the first approximation to the standard error of λ, the maximum likelihood estimate of the bacterial density, λ. Substituting $\hat{\lambda}$ for λ in this formula, he tabulated (together with the values of $\hat{\lambda}$) for certain combinations of tubes of dilutions the corresponding estimates of the standard deviation of $\hat{\lambda}$. In two subsequent papers (94, 96), he studied the effect on the standard error of $\hat{\lambda}$ and on the coefficient of variation of $\hat{\lambda}$ of varying: (a) the number of tubes used at each dilution, (b) the true bacterial density, and (c) the number and type of dilutions used. He found that all three factors must be taken into account in determining the accuracy of $\hat{\lambda}$. For low densities, *e.g.*, 20 organisms per 100 cc, he points out that an equal number of tubes at the dilutions 1/2, 1/10, 1/100 provides more accurate results than the same number of tubes at each of the dilutions 1/10, 1/100, 1/1000. To facilitate the use of the former dilution system he tabulated (95) the estimates $\hat{\lambda}$ and their estimated standard errors when 2, 3, 5, or 10 tubes are employed at each dilution.

In connection with Swaroop's results two points need to be kept in mind. First, the formula employed for the standard error of $\hat{\lambda}$ is strictly valid only when a large number of tubes are employed, giving merely a first approximation of *unknown accuracy* when only a few tubes are involved. Second, the standard error of a quantity is a good measure of its sampling variability only when the distribution of the quantity is approximately normal (Gaussian). To what extent these may impose limitations in actual practice can be inferred from direct calculations carried out by Halvorson and Ziegler (50, 51). For the case of ten tubes at each of three dilutions in geometric progression with (ascending) factor of 10, Halvorson and Ziegler (51) calculated, from the terms of an appro-

priate multinomial expansion, the exact probabilities of observing the various possible codes when the true density was 0.15, 0.25, 0.50, and 1.50 per cc, and then, with the aid of their tables showing the correspondence between codes and estimates, they obtained the probability distribution of the maximum likelihood estimator, $\hat{\lambda}$, when the true density was each of the four preceding values. They found: (a) that the distributions of $\hat{\lambda}$ in these cases were moderately skewed with

TABLE 5

Comparison of arithmetic and logarithmic estimation

NOTE	FORMULATION	ACTUAL BACTERIAL DENSITY PER CC (λ)			
		0.15	0.25	0.50	1.50
(i)	$M(\hat{\lambda})$	0.164	0.284	0.558	1.648
(ii)	$\sigma(\hat{\lambda})$	0.0659	0.1171	0.2263	0.6894
(iii)	$\sigma(\hat{\lambda})/\lambda$	0.440	0.468	0.453	0.460
(iv)	$\text{Lim } \sigma(\hat{\lambda})/\lambda$	0.354	0.353	0.407	0.357
(v)	$[M(\hat{\lambda}) - \lambda]/\sigma(\hat{\lambda})$	0.212	0.290	0.256	0.215
(vi)	$\log \lambda$	−0.824	−0.602	−0.301	+0.176
(vii)	$M(\log \hat{\lambda})$	−0.816	−0.578	−0.285	+0.184
(viii)	$\sigma(\log \hat{\lambda})$	0.163	0.164	0.163	0.168
(ix)	$\text{Lim } \sigma(\log \hat{\lambda})$	0.1535	0.1532	0.1768	0.1550
(x)	$\dfrac{M(\log \hat{\lambda}) - \log \lambda}{\sigma(\log \hat{\lambda})}$	0.049	0.146	0.098	0.048

Notes:

(i) The arithmetic mean of the distribution of the maximum likelihood estimator, $\hat{\lambda}$, directly from table 1 of Halvorson and Ziegler (51).

(ii) The standard deviation of $\hat{\lambda}$, *i.e.*, the root mean square deviation from $M(\hat{\lambda})$, taken table 1 of Halvorson and Ziegler (51).

(iii) Entries obtained by dividing those in (ii) by λ, and *not* equal to Halvorson and Ziegler's coefficient of variation from mean, since their divisor is the corresponding *entry* in (i).

(iv) The limiting value of $\sigma(\hat{\lambda})/\lambda$ as the number of tubes at each dilution increases indefinitely, taken from table 2 of Swaroop (94).

(v) Obtained from the preceding rows of the present table.

(vi) The logarithm (to base 10) of the actual bacterial density per cc.

(vii) The arithmetic mean of $\log \hat{\lambda}$, taken from table 1 of Pearson (74) who calculated them from table 1 of Halvorson and Ziegler.

(viii) The standard deviations of $\log \hat{\lambda}$ taken from Pearson's table 1, who *calculated* them from table 1 of Halvorson and Ziegler.

(ix) The limiting value of $\sigma(\log \hat{\lambda})$ as the number of tubes at each dilution increases indefinitely, taken from Pearson's table 1 who calculated them from the *formula of Fisher* (28).

(x) Calculated from the preceding rows of the present table.

the long tail toward the large values; (b) that the degree of *skewness* was practically independent of the true density, λ, for the range of λ considered; (c) that the standard deviation of $\hat{\lambda}$, $\sigma(\hat{\lambda})$, increased with λ; and (d) that the coefficient of variation, $\sigma(\hat{\lambda})/\lambda$, was practically constant for the range of λ considered. Some of their results are given in table 5, together with additional results derived from their exact distributions of $\hat{\lambda}$ and from Fisher's formula.

From this table it is evident *for the density range considered*: (a) that the standard deviation of $\hat{\lambda}$ varies with λ, being to a good approximation equal to $0.45\,\lambda$; (b) that the standard deviation of log $\hat{\lambda}$ is nearly constant; (c) that log $\hat{\lambda}$ and $\hat{\lambda}$ both have a positive bias, and in standard deviation units the bias of the former is somewhat less; and (d) that the relative error in using the limiting standard deviation in place of the exact value is less with log $\hat{\lambda}$ than with $\hat{\lambda}$. Pearson (74) gives a table of values for the limiting standard deviation of log $\hat{\lambda}$ for λ from 0.10 to 4.00, and from a graphical portrayal of these values it appears that the limiting standard deviation of log $\hat{\lambda}$ ranges from 0.151 to 0.180, with a median value of approximately 0.166. Since the distributions of log $\hat{\lambda}$ are more nearly symmetrical than the distributions of $\hat{\lambda}$, it is of interest to see how well the distributions of log $\hat{\lambda}$ can be approximated by assuming that it is normally distributed with a standard deviation of 0.166 about log λ. Using this approximation, the probability should be 0.95 that $\hat{\lambda}$ will lie in the intervals 0.07–0.32, 0.118–0.723, 0.236–1.058, 0.671–3.158 (end points included) when $\lambda = 0.15$, 0.25, 0.50, and 1.5, respectively. From Halvorson and Ziegler's table 1 the exact probabilities appear to be 0.949, 0.986, 0.948, 0.937, respectively. The agreement is remarkably good, especially in the first and third cases, in view of the fact that $\hat{\lambda}$ in each instance can take only *certain* discrete values, the probabilities of which do *not* increase monotonically but exhibit many vicissitudes as $\hat{\lambda}$ approaches the true value, λ, from either side.

Remarks and Suggestions. To remind a bacteriologist of the many statistical procedures which have been advocated for solving the type problem represented by the dilution count may not strike him as entirely a favor. He may, indeed, find himself somewhat distracted by the variety of aid offered, his position not unlike that of Joel Chandler Harris's Br'er Fox hesitating to decide which barbecue to attend. The bacteriologist of this joint undertaking, therefore, put these two questions to the statistician:

1. Which of the proposed methods should I employ in a given situation at the present time, *i.e.*, with only existing tables and charts to facilitate their application?

2. If funds were available, what further research should be undertaken to advance statistical methodology relating to the dilution count?

It is realized that the following answer of the statistician represent "one man's opinion" and is to be accepted or rejected as such.

In order to answer the first question, a more specific definition of the situation is desirable. If a routine check on a controlled bacterial population is concerned, as in a routine analysis of the water in a city reservoir, so that the customary whereabouts of the bacterial density is known, the most effective 'control' will be obtained by running all tubes at a single dilution, the dilution and number of tubes being chosen in such a way that the probability of obtaining a proportion of positive tubes which will result in condemnation will be very small when the bacterial density is within the customary neighborhood, and large when the bacterial density exceeds the permissible limit. The standard proposed in the Report to which Reed's (78a) analysis is an appendix is a step in this direction.

To improve the chances of a routine analysis detecting the entrance of trouble before it leads to condemnation, the routine should include the use of a control chart in terms of the *proportion* of positive tubes, as outlined in American War Standard Z1.3.[11]

If on the other hand, an isolated analysis or a routine analysis of an uncontrolled bacterial population is concerned, as in an analysis of stream water prior to treatment at the Water Works, the general whereabouts of the bacterial density being unknown in advance, then 10 tubes should be run at each of a series of dilutions in geometric progression with (ascending) ratio of 10. A single estimate of the bacterial density can readily be obtained from the table of Halvorson and Ziegler (48, 49); its confidence limits can be estimated from a chart[22] prepared by Miss Supińska (91). In the absence of Miss Supińska's chart, confidence intervals computed from $\log \hat{\lambda} \pm (1.96) (0.166)$ can be used, the associated confidence coefficient being close to 0.95.

Table 6 gives, for three hypothetical cases discussed by Gordon (41), the maximum likelihood estimate, $\hat{\lambda}$, tabulated by Halvorson and Ziegler; the estimate recommended by Gordon, $\bar{\lambda}$; and the upper and lower limits to 0.95

TABLE 6
Estimation by interval and by single value

CODE	$\hat{\lambda}$	$\bar{\lambda}$	Supínska		Normal Approximation		Matuszewski, et al.	
			λ min.	λ max.	λ min.	λ max	λ min.	λ max.
10–7–3	1.53	1.43	0.67	3.00	0.72	3.24	0.75	4.28
8–5–1	0.267	0.291	0.125	0.525	0.126	0.563	0.179	1.153
4–2–1	0.080	0.086	0.029	0.165	0.041	0.182	0.034	0.203

confidence intervals obtained (a) by Supińska's method, (b) by the normal approximation discussed above, and (c) by a method described by Matuszewski, et al. (67) which depends only upon the sum of the three components of the code. The intervals obtained by normal approximation agree moderately well with

[22] This chart has been reproduced by Matuszewski, Neyman, and Supińska (67). It was not based on an exact mathematical solution, but was obtained by graduating a series of experimental sampling results as described on p. 76 of their paper, *viz.*,

"The method followed by Miss J. Supińska consisted in a complex sampling experiment, using Tippett's random sampling numbers. The experiment produced a series of values of the variates x_0, x_1, and x_2 [i.e., codes $x_0-x_1-x_2$] following the sampling distribution which they would follow in our hypothetical conditions of the experiment. For each series of x_0, x_1, and x_2, it was possible to read up from the table of Halvorson and Ziegler an estimate, say λ', of the concentration λ. The estimates λ' have been then tabulated and an empirical frequency distribution of λ' corresponding to several fixed values of λ has been determined. Following the method described by J. Neyman, these empirical frequency distributions were then used to construct confidence intervals as if they were the accurate ones. As the random variation could not fail to affect the limits of the intervals it was felt necessary to correct them by fitting two parabolae, one marking the lower and the other the upper limits of the confidence intervals."

Miss Supińska's intervals, and in the absence of her chart will probably be close enough for most practical purposes. It will be noted that they are slightly wider and displaced somewhat to the right. This displacement is due in part to the fact, noted above, that log $\hat{\lambda}$ tends to overestimate log λ slightly—*e.g.*, when $\lambda = 1.50$, the mean of log $\hat{\lambda}$ is 0.184, the antilogarithm of which is 1.53— and a correction for this bias might be devised.

Of these three sets of intervals, those taken from the table of Matuszewski, *et al.*—whose paper provides an excellent introduction to the construction and interpretation of confidence intervals—are the only ones which are based on an exact mathematical solution. The mathematical approach which they have adopted, is, essentially, an extension of a short-cut proposed by Fisher (28) whereby the estimation is based solely upon the sum of the components of the code. Thus the codes 10-10-0, 10-9-1, $\cdots$, 10-7-3, $\cdots$, 10-5-5, 9-9-2, $\cdots$ yield the same single estimate by Fisher's short-cut method, and lead to the same confidence interval by the corresponding method of Matuszewski, *et al.*, whereas they lead to quite different values of $\hat{\lambda}$ in the table of Halvorson and Ziegler and thence to quite different confidence intervals from Miss Supińska's chart. The confidence intervals of Matuszewski, *et al.* are mathematically rigorous but they utilize only a portion of the information in the data, and hence are not to be recommended for accurate work. In the absence of Halvorson and Ziegler's table or Miss Supińska's chart, they will provide broad interval estimates of λ which will be correct in the long run, in at least 95 per cent of the instances in which they are used.

In two recent papers Halvorson and his associates (47, 80) considered the use of too frequent occurrence of rare codes for diagnosing the character of departures of laboratory technique from statistical control. Stein (88) gave a graphical means of checking the consistency of the results obtained at each of two dilutions differing by a factor of 10 when an equal number of tubes were run at each dilution, and his method can be extended to an equal number of tubes at each of three dilutions. Perhaps these two approaches can be combined to give a rapid graphical test of the statistical control of the experimental technique in terms of the consistency of the results of successive dilutions.

With regard to further research, it seems highly desirable to construct mathematically exact charts giving 0.95 and 0.99 confidence intervals for the bacterial density for the case of several tubes at a single dilution, and for the case of one or more tubes at each of the successive dilutions. For several tubes at one dilution, such charts can easily be constructed from Clopper and Pearson's (18) chart of confidence intervals for the binomial distribution, or from Fisher and Yates' table of fiducial limits for the binomial distribution.[23] When a sufficient number of exact frequency distributions of $\hat{\lambda}$, such as are given in Table 1 of Halvorson and Ziegler (51) have been computed, exact confidence charts patterned after Miss Supińska's empirical chart can be constructed for the case of 10 tubes at each of three successive dilutions in geometric progression with

[23] We understand that the second edition of Fisher and Yates (35) scheduled for 1942 publication includes such a table.

(ascending) factor of 10. Charts of this kind not only provide confidence limits for λ, given $\hat{\lambda}$, but for any particular (*e.g.*, maximum permissible) value of λ the charts can be used also to obtain control limits for $\hat{\lambda}$ such that, if $\hat{\lambda}$'s lying outside the limits are interpreted as danger signals, there will be a small known probability of an unwarranted "Wolf! Wolf!" While the limits corresponding to 10 tubes at each of the (three) successive dilutions with (ascending) dilution factor of 10 would probably be most widely used, the limits for 5 tubes at each dilution might be included on the same chart to show the effect of reducing the number of tubes on accuracy of estimation. Similar charts could be constructed for the McCrady-Swaroop system. A comparison of these charts would bring out at once some of the relative advantages and disadvantages of the two systems.

THE NORMAL DISTRIBUTION

It is evident that the graph of a binomial distribution consists of a series of bars of appropriate lengths erected at the points $x = 0, 1, 2, \cdots, n$, that they are always spaced at intervals of 1 unit whatever the value of n, and that the number of these bars increases as n increases. When n becomes only moderately large, say 25, evaluation of successive terms of the point binomial becomes laborious, and increasingly so as n increases. Furthermore, since the sum of the terms of $(q + p)^n$ is always unity for $0 < p < 1$ and $q = 1 - p$, the values of the respective terms, and hence the lengths of the corresponding bars, must decrease toward zero as n increases. In other words, whatever the value of p, the probability of the event occurring in any single trial, the probability of its occurring *exactly x times* in n independent trials will be practically zero when n is very large. Therefore, what is generally sought in practical work is *not* the probability that an event will occur exactly 600 times in 1000 independent trials, but the probability that it will occur between 525 and 650 times, or the probability that it will occur *at least* 600 times. In short, for large values of n, what is needed is a convenient method for summing the appropriate terms of a point binomial.

The normal probability curve, whose equation can be written

$$[8] \qquad\qquad y = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-m)^2}{2\sigma^2}}$$

where m and σ are the parameters of the distribution, was developed by Abraham De Moivre (23) in 1733 as a means of finding easily the sum of consecutive terms of a binomial distribution when n is large. He showed that for large values of n the term involving p^x in the expansion of $(q + p)^n$, *i.e.*, the term which gives the probability of the event occurring *exactly x times* in n independent trials, is well *approximated* by equation 8 with $m = np$ and $\sigma^2 = npq$; and that the sum of the terms corresponding to values of x from X_1 to X_2 *inclusive* where $0 \leq X_1 \leq X_2 \leq n$ is given *approximately* by the area under the curve between $x = X_1 - 1/2$ to $x = X_2 + 1/2$ when $m = np$ and $\sigma = \sqrt{npq}$. For the approximation to be good, n should be large and $p - q$ should be numerically small

compared with $\sqrt{npq}$. Nevertheless, the approximation is fairly good even when n is as low as 10 and $p = 1/3$, as is shown in Table 7. The fit for $p = 1/2$ is much better, of course.

Until quite recently (76), this early work of De Moivre (23) had been overlooked, and the development of the normal distribution was often attributed to Gauss, who proposed its use as the distribution of errors of observation arising in astronomical work. Gauss discussed in detail the adjustment of measurements for errors of observation and laid the foundations of the method of least squares so thoroughly that many of the techniques employed today are essentially those of Gauss. Throughout his work the 'errors' were regarded as normally distributed random variables; equation 8 written in slightly different

TABLE 7

Comparison of binomial expansions with corresponding normal distribution curves

NUMBER OF "SUCCESSES"	$1000 \left(\frac{1}{2} + \frac{1}{2}\right)^{10}$		$1000 \left(\frac{1}{3} + \frac{2}{3}\right)^{10}$	
	Binomial expansion	Normal curve	Binomial expansion	Normal curve
0	1.0	2.2	17.3	28.7
1	9.7	11.2	86.7	80.6
2	44.0	43.5	195.1	178.8
3	117.2	114.4	260.1	256.5
4	205.1	204.7	227.6	238.6
5	246.0	248.0	136.6	143.8
6	205.1	204.7	56.9	56.2
7	117.2	114.4	16.3	14.3
8	44.0	43.5	3.1	2.3
9	9.7	11.2	0.3	0.2
10	1.0	2.2	0.0	0.0
	1,000.0	1,000.0	1,000.0	1,000.0

$1000 \left(\frac{1}{2} + \frac{1}{2}\right)^{10}$ might represent the distribution of the number of heads obtained per throw if 10 coins are tossed 1000 times; the "binomial expansion" gives the theoretical values obtained by expanding the binomial; the "normal curve" represents those obtained from the normal curve corresponding to this binomial, *i.e.*, where $m = np = 5$ and $\sigma = \sqrt{npq} = \sqrt{10/4}$.

Similarly $1000 \left(\frac{2}{3} + \frac{1}{3}\right)^{10}$ could represent the distribution of 'successes' when 10 dice are thrown 1000 times and a 'success' consists of *either ace or six* appearing on a die.

form, was referred to as the *law of errors* or the *error function*, names which it still retains in the physical sciences. Because of its widespread use in the theory of errors, the normal distribution is often called the *Gaussian Distribution*. Laplace developed the normal distribution as an approximation to the binomial distribution when n is large, apparently unaware of De Moivre's earlier development, and applied it to a variety of phenomena, especially to games of chance and vital statistics. In his analytical treatise on probability, Laplace (59) laid the foundations of modern mathematical statistics.

Adolphe Quetelet (1796–1874) led the way in applying the normal curve to biological and social phenomena, and Francis Galton (1822–1911) applied it to biological variables of every sort. Both were impressed by the way their data seemed to conform to this curve. There appears to have been a belief among many biologists of this period that this curve was an ideal to which

most biological distributions ought to conform, and that some explanation was needed when they did not. In consequence, the expressions "normal law" and "normal distribution" took root as substitutes for "law of error", "Gaussian distribution", etc. However, as more and more data were studied, and better methods of comparison were developed, it became evident, principally through the work of Karl Pearson (1857–1936), that the normal distribution is not a universal law of nature.

The practical importance of the distribution has not declined, however, on this account, but has actually increased in importance in recent years. The principal reasons for this are:

(a) Even though a population departs radically from normality, a secondary population formed of the arithmetic means of sufficiently large random samples drawn from it can be regarded without sensible error as normally distributed about the mean of the parent population with a variance equal to that of the parent population divided by the size of the sample. Hypothetical populations can be devised for which the preceding statement is false, but it may be considered true for biological populations, since biological variables take on finite values only. How large the samples need to be depends largely on the asymmetry of the population; for symmetrical and only moderately asymmetrical *continuous* distributions, samples of 4 are often large enough and samples of 10 are quite adequate, but with very skewed distributions somewhat larger samples may be needed.

(b) The normal distribution has many mathematical properties which make particularly attractive in the development of statistical theory and techniques; ence most new techniques are developed on the supposition that the underlying variables are normally distributed.

(c) The assumption of an underlying normal distribution does not lead to serious error, since many such techniques are based on statistics and test criteria whose sampling distributions are relatively stable for moderate departure of the true underlying distribution from normality, especially if the sample is large.

Since different normal distributions are obtained by assigning different values of m and σ in equation 8, it is advantageous for purposes of tabulating properties (e.g., the values of y corresponding to a given value of x, the area under the curve between two points, etc.) to standardize this family of curves by reducing all to a single form. This is accomplished by substituting for x a new variable defined by

[9] $$u = \frac{x - m}{\sigma}$$

which reduces equation 8 to

[10] $$y = \frac{1}{\sqrt{2\pi}} e^{-\frac{u^2}{2}}$$

The area under the curve of equation 8 between $x = X'$ and $x = X''$ is equal to the area under the curve of equation 10 between $u = u'$ and $u = u''$, where

after that the power of these tests relative to a fixed alternative increases rapidly with the number of observations employed.

DATA FROM NORMAL DISTRIBUTION

To illustrate the use of the normal distribution in tests of significance let us consider the following data which represent milligrams of yeast produced in two media.

	Medium A	Medium B
	100	85
	110	96
	85	74
	90	80
	93	83
Total	478	418
Average	95.6	83.6

Example 1: Suppose past experience has shown that an individual estimate of yeast growth by the method used has a standard deviation of 10 milligrams and that on the average a yield of 90 milligrams is obtained in a certain standard medium. We ask then: Are the observed values consistent with the hypothesis that medium A is equivalent to the standard medium? That is, are the observed values in medium A independently and normally distributed about an expected value (m) of 90 with a standard deviation (σ) of 10. The admissible alternative is that the observed values are independently and normally distributed with a σ of 10 about a mean different from 90.

To test this hypothesis the normal deviate

$$11]\qquad x = (\bar{X} - m)/(\sigma/\sqrt{N}) = (95.6 - 90.0)/(10/\sqrt{5}) = 1.25$$

is calculated from the data, N and $\bar{X}$ denoting the number of and average value of the observations, respectively. The deviate found in the present instance being numerically less than the 5 per cent significance level,[24] 1.96, the hypothesis may be accepted tentatively, that is, we tentatively conclude that medium A is equivalent to the standard medium for propagation of yeast.

Since in this example the number of observations involved is very small, the test has little discriminating power with respect to nearby values of m. It may be shown, for instance, that for $N = 5$ the above test conducted at the 5 per cent level of significance has slightly better than a 0.50 chance of rejecting $m = 90$ when in fact $m = 80$ or $m = 100$.[25] On the other hand, for $N = 16$ and

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[24] See, for instance, Fisher (30) table 1.

[25] It may be noted here that, if the test were conducted at the 1 per cent level of significance, the probability of rejecting $m = 90$ when $m = 80$ or $m = 100$ would be only 0.27, so that the probability of an error of the second kind would be 0.73 in these instances. This illustrates a general principle: reduction of the probability of an error of the first kind by choosing a more stringent level of significance increases the probability of an error of the second kind.

$\sigma = 10$ the probability that the test will reject $m = 90$ when in fact $m = 80$ or $m = 100$ is approximately 0.98 when the test is conducted at the 5 per cent level of significance. It follows from this that with very small numbers of observations the chances of committing an error of the second kind are generally great. Otherwise stated, if 50 or more observations had yielded the above deviate, 1.25, we should have greater confidence that m was actually close to 90.

Example 2: Next, consider the question whether media A and B are equivalent. To do this, we test the hypothesis that the data for the two media represent independent random samples from normal populations with standard deviation $\sigma_A = \sigma_B = 10$, and equal means $m_A = m_B$, the admissible alternatives being that the data represent independent random samples from normal populations with $\sigma_A = \sigma_B = 10$ but unequal means $m_A \neq m_B$.

To test this hypothesis the normal deviate

[12] $x = (\bar{X}_1 - \bar{X}_2)/\sigma(1/N_1 + 1/N_2)^{\frac{1}{2}} = (95.6 - 83.6)/10(2/5)^{\frac{1}{2}} = 1.90$

is calculated from the data. N_1 and N_2 are the number of observations from A and B, respectively, $\bar{X}_1$ and $\bar{X}_2$, the corresponding averages, and σ, the postulated common standard deviation. The deviate obtained in the present instance is close to, but does not exceed the 5 per cent level, 1.96, so that at this significance level the hypothesis tested should be tentatively accepted, and we might conclude that the two media are essentially equivalent. In practice, however, one would be reluctant to place great confidence in the conclusion that the data represent independent random samples from identical normal distributions. First, as already noted, the chances of an error of the second kind are great with so few observations. Second, the value obtained for the deviate is highly dependent on the value taken for $\sigma_A = \sigma_B$. With the new media, for example, it might be that $\sigma_A = \sigma_B = 8$, and this would lead to a deviate of 2.37. Since this value exceeds the 5 per cent significance level by a good margin, we should reject that portion of the hypothesis which states that $m_A = m_B$, had we postulated a standard deviation of 8.

Example 3: Although we might not place much confidence in the assumption that both σ_A and σ_B are equal to the σ observed with the standard medium, theoretical considerations might suggest the less restrictive assumption that σ_A and σ_B are equal. If so, "Student's" t test can be used. Suppose we ask the same question as in Example 2 but make no assumptions about the true value of $\sigma_A = \sigma_B$. Formally, we state this: Test the hypothesis that the data for media A and B constitute independent random samples from identical normal populations, the admissible alternatives being that the two parent populations are normal with identical standard deviations but different means.

We calculate the statistic,

[13] $$t = (\bar{X}_1 - \bar{X}_2)/s(1/N_1 + 1/N_2)^{\frac{1}{2}}$$

where

[14] $$s^2 = [\Sigma(X_1 - \bar{X}_1)^2 + \Sigma(X_2 - \bar{X}_2)^2]/(N_1 + N_2 - 2).$$

X_1 and X_2 represent individual observations from A and B, respectively; the other symbols have the same meanings as in equation 12. This test, developed by Fisher (31), is an extension of a result obtained earlier by "Student" (90). The probability distribution of t depends on its degrees of freedom, which here are $N_1 + N_2 - 2$; tables of the significance levels of the distribution are available (30, 35, 79, 84).

For purposes of calculation it is often convenient to evaluate the summations from

$$\Sigma(X - \bar{X})^2 = \Sigma X^2 - (\Sigma X)^2/N$$

[15] $\Sigma(X_1 - \bar{X}_1)^2 = 100^2 + 110^2 + 85^2 + 90^2 + 93^2 - 478^2/5 = 377.2$

$\Sigma(X_2 - \bar{X}_2)^2 = 261.2$

therefore,

$$s^2 = (377.2 + 261.2)/8 = 79.80, \quad \text{and}$$
$$t = (95.6 - 83.6)/\sqrt{79.8(2/5)} = 2.12$$

This value does not exceed the 5 per cent point, which for 8 degrees of freedom is 2.306, so that at this level of significance the hypothesis tested may be accepted tentatively, i.e., the two media are equivalent. The risk of an error of the second kind discussed in connection with Example 2 has equal relevance here.

Example 4: If the method of obtaining the data is such that there exists a pairing of the values, the foregoing tests should not be employed. Instead, one should base the test on the successive differences between the pairs, testing whether the true mean difference is zero. For example, if in the yeast experiment the values 100 and 85 were determined in one run, 110 and 96 at another and so on, we test the hypothesis that the *differences* may be regarded as independent random observations from a normal population with *zero* mean, the admissible alternatives being that the *differences* are independent random observations from a normal population with non-zero mean.

The appropriate t to use to test this hypothesis is

[16] $$t = \bar{d}/(s/\sqrt{N})$$

where

[17] $$d = X_1 - X_2, \qquad \bar{d} = (\Sigma d)/N = \bar{X}_1 - \bar{X}_2$$

[18] $$s^2 = \Sigma(d - \bar{d})^2/(N - 1),$$

and the degrees of freedom are $N - 1$.

In the present instance $t = 12/(2.3452/\sqrt{5}) = 11.44$ which greatly exceeds the 1 per cent level (4.604) for 4 degrees of freedom. Accordingly, if in the long run, one is willing to risk committing an error of the first kind less than 1 time in 100, the discrepancy between observation and hypothesis in the present instance may be regarded as sufficient to warrant rejection of the hypothesis tested.

The hypothesis is rejected as a whole and further consideration is necessary

before one particular aspect is blamed. In the present instance, we have focussed our attention primarily on the equivalence of the media for yeast propagation and would, accordingly, be inclined to accept the alternative that the true mean difference was not zero, *i.e.*, medium A is superior to medium B. By construction, the t test is most powerful with respect to this class of alternatives, so that this explanation is generally the one to be adopted. But other alternatives should not be disregarded, such as: (a) non-randomness of sampling; (b) lack of independence of the successive differences; (c) non-normality of the common population. Fortunately, as a test of the hypothesis that the true mean difference is zero, the t test is relatively insensitive to moderate departures from normality. The issues of randomness and independence can be taken care of in the design and conduct of the research. It is for this reason that it is always desirable to introduce an element of randomization in experimental design. Similar remarks apply to the rejection of the hypotheses discussed in Examples 1 to 3.

When more than two samples of the data are at hand, pair-wise comparisons among all possible pairs with t tests do not provide a satisfactory method of testing the hypothesis that they are all from the same normal population, with the alternatives that their true means may differ. *Analysis of variance* provides the tests which are the extensions of t tests appropriate to such cases.

ANALYSIS OF VARIANCE AND DESIGN OF EXPERIMENTS

In applied phases of agricultural research the experimenter must frequently overcome handicaps which quantitatively at least appear more formidable than the corresponding ones of the laboratory scientist. These include:

1. Heterogeneity of the experimental material—this arises for example, from wide differences in soil fertility or pronounced variation in stock animals.

2. Restriction of replication—because of the expense and other considerations, the size and number of experimental plots or replicate animals is definitely limited.

3. Length of experiment—an experiment usually lasts through a growing season or reproductive period so that one or two experiments a year is the most one can hope to make. Moreover, because of unfavorable weather, depredation of animals, or of other accidents, the entire experiment may be lost.

It is understandable then that the complex type of experiment early found favor with the agronomist and animal husbandryman—if experimentation is limited, as many variables as practical should be included in order to gain maximum information when the experiment succeeds. The meaning of data from such experiments, however, was very obscure, and it was not until Fisher and his group at the Rothamsted Experimental Station developed the methods for analysis of variance that any real basis for comprehensive interpretation became available.

Although the particular handicaps referred to may not bother the laboratory scientist, their counterpart is constantly with him in various guises, and it would be unfortunate if he overlooked the powerful tools now at his disposal for analysis of data merely because the tools were originally developed for field trials. The principles used are perfectly general

and are equally applicable to data from investigations on pig feeding, fertilizer treatment, or bacterial nutrition.

Unfortunately, the exposition of the subject has been confined almost entirely to examples taken from field plots, animal production, or plant and animal breeding studies. Consequently, scientists unacquainted with the particular terminology or type of problem of such investigations may obtain only a hazy idea of the real nature of the analysis and may *be inclined to regard* the entire matter as a somewhat mysterious hocus-pocus useful only in applied agriculture where uncontrollable factors and lack of replication prevent use of the precise techniques of the laboratory. Actually, the development of the analysis of variance has allowed the introduction of the complex experiment, or "factorial design" as it is nowadays called, into other branches of science rather than restricting it to applied studies where it was formerly tolerated only because nothing better was at hand. The advantage of the complex experiment is not only its efficiency, *i.e.*, its economy of time and expense for a given amount of information, but also its insistence on close attention to experimental design and its ability to bring out information through the 'interaction' terms which either cannot be obtained or only with great effort in the single factor type of study. *More often than not, the interactions are precisely the information wanted if the results are to have significance for conditions other than the carefully standardized ones of the experiment.* (See Chapter VII in Fisher's *Design of Experiments* (33) for further discussion of the technical advantage of factorial design.)

Fisher suggests, ". . . perhaps it is worth while stating an impression I have formed—that the analysis of variance, which may perhaps be called a statistical method, because the term is a very ambiguous one—is not a mathematical theorem, but rather a convenient method of arranging the arithmetic." (Discussion of Wishart's (118) paper.) Although this is probably a modest understatement, it is a point of view that should be kept in mind for an understanding of the steps taken in an analysis. These may be enumerated:

1. A *total* sum of squares is calculated by squaring the deviations of the respective observations from their common mean without any attention being paid to treatments, strains, or other factors.

2. This sum of squares is divided into its components, thus assigning to each factor *in the experiment its proper share of the observed variation* among the observations.

3. A variance estimate from each factor is determined by dividing its share of the sum of squares by the appropriate degree of freedom.

4. The observed inequalities between certain of the variance estimates are tested for significance.

The general nature of the analysis of variance can be appreciated by noting certain properties of an arbitrary set of numbers arranged in k groups with n numbers in each group:

	Observation number							Total	Mean
	1	2	3		j		n		
1	x_{11}	x_{12}	x_{13}	$\cdots$	x_{1j}	$\cdots$	x_{1n}	T_1	$\bar{x}_1$
2	x_{21}	x_{22}	x_{23}	$\cdots$	x_{2j}	$\cdots$	x_{2n}	T_2	$\bar{x}_2$
	$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$								
i	x_{i1}	x_{i2}	x_{i3}	$\cdots$	x_{ij}	$\cdots$	x_{in}	T_i	$\bar{x}_i$
	$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$								
k	x_{k1}	x_{k2}	x_{k3}	$\cdots$	x_{kj}	$\cdots$	x_{kn}	T_k	$\bar{x}_k$
								T	$\bar{x}$

group

If the mean of the entire group is $\bar{x}$, and $\bar{x}_i$ is the mean for the ith group, it can be shown that the following equation holds:

$$[19] \qquad \sum_{i=1}^{k} \sum_{j=1}^{n_i} (x_{ij} - \bar{x})^2 = \sum_{i=1}^{k} \sum_{j=1}^{n_i} (x_{ij} - \bar{x}_i)^2 + \sum_{i=1}^{k} n_i(\bar{x}_i - \bar{x})^2.$$

That is, the sum of the squares of the deviations of the individual numbers (x_{ij}) from their general mean can be divided into two parts: (1) the sum of the squares of the deviations of the numbers in each row from the mean of that row summed for all the rows; (2) the sum of the squares of the deviations of each row mean from the general mean multiplied by the number of items in the row. In our example the rows have an equal number, but this is not a necessary restriction.

So far nothing has been said about the nature of the numbers since equation 19 holds independent of what they represent. Now let us consider them to be results of an experiment in which k treatments have been used and each treatment has been represented n times. The first term on the right hand side when divided by $k(n - 1)$ provides an estimate of σ^2 from variation *within the treatments*; the second term divided by $(k - 1)$ provides an estimate of σ^2 from variation *between treatment means*. If the k samples (k sets of n determinations each) are from normal populations with equal variances, *i.e.*, $\sigma_1^2 = \sigma_2^2 = \sigma_3^2 = \cdots = \sigma_k^2 = \sigma^2$, then a test of whether the means of these populations are equal can be devised. For if the variance estimate from *between treatment means* is denoted by A and the variance estimate from *within treatments* by B, it can be demonstrated that A and B are both unbiased estimates of σ^2 when the true treatment means are identical. It remains only to find a method for testing whether any two estimates of a variance, both subject to sampling errors, are estimates of the same true variance, σ^2. This is done by defining a new variable, $F = A/B$, and determining its distribution when A and B are both estimates of the same variance. As would be expected, this distribution depends on the values of $(k - 1)$ and $k(n - 1)$, the respective degrees of freedom of A and B. From the distributions of F, one can determine the probability that a given value of F will be exceeded through chance fluctuations when A and B are estimates of the same variance. Such information is condensed in tables, so that an observed value of F can be readily tested for significance by consulting the table under the proper degrees of freedom (84).

A few examples of varying degrees of complexity will illustrate how the method is used. The following data were obtained in a nitrogen fixation experiment:

CULTURE	N IN MILLIGRAMS			T_i
A	61	36	71	168
B	39	46	42	127
C	17	28	34	79
D	22	15	43	80

The triplicate determinations are not paired in any way through design of experiment. Is there reason for concluding that the mean nitrogen contents of these cultures differ? Our 'null' hypothesis (see Appendix) is that the samples are from the same population. The question asked may be rephrased in statistical terminology: Do the independent estimates of the variances from *between* and *within* treatments differ significantly? To answer this, the following estimates of variance are calculated: The *total sum of squares* is determined from equation 15 with the summation extending over all N observations and with $T = \Sigma X$

$$\Sigma(X - \bar{X})^2 = \Sigma X^2 - \frac{T^2}{N} = 61^2 + 36^2 + 71^2 \cdots 22^2 + 15^2 + 43^2 - 454^2/12$$

$$= 3069.7$$

Then, the *sum of squares between treatments* (different cultures)

$$[20] \quad \sum_{i} n_i(\bar{X}_i - \bar{X})^2 = \sum_{i} \frac{T_i^2}{n_i} - \frac{T^2}{N}$$

$$= (168^2 + 127^2 + 79^2 + 80^2)/3 - 454^2/12 = 1821.7$$

Having calculated these sums of squares in this simple case, we can set up the analysis immediately since the sum of squares *within treatment* is the difference between the *total* and that *between treatments*.

VARIATION	D.F	SUM OF SQUARES	MEAN SQUARE	F	5% POINT
Between treatments	3	1,821.7	607 2	3 89	4 07
Within treatment (error)	8	1,248	156 0		
Total	11	3,069.7			

From the table we find that with 3 and 8 degrees of freedom, F may be expected to be at least 4.07, 5 times in 100 through chance alone, when the samples are from populations having a common mean and variance. Therefore, the observed differences in the estimates of variance are not large enough to be significant at the 5 per cent level, and we may conclude that these data do not conflict with our null hypothesis: that the samples are from the same population. It should be observed that in this simple case, the analysis of variance is merely an extension of "Student's" t test for the difference between two means. Actually, each of the means could be tested against the others by this method, but by combining the data in an analysis of variance we obtain greater precision since our estimate of error is now based on 8 degrees of freedom instead of 4 as would be the case if the t test were used. This is one of the main advantages of the design suggested by the analysis of variance, *viz.*, that in a given experiment, more treatments with fewer replications can be used, because the estimate of error will be based on all the samples, not merely on those of the two particular means which are to be compared.

Consider now a slightly more complex type of experiment in which two factors are varied. The following data from Thorne, Neal, and Walker (100) summarize the respiratory quotients of different species of the root nodule bacteria growing in a basic medium in which the source of nitrogen was varied:

SOURCE OF NITROGEN	R. MELILOTI	R. TRIFOLII	R. LEGUMINOSARUM	R. JAPONICUM	R. PHASEOLI	MEAN	T_i
Sodium nitrate.	1.21	1.15	1.03	1.03	1.12	1.10S	5.54
Ammonium chloride..	1.1S	1.15	1.07	1.0S	1.13	1.12	5.61
Asparagin . . .	1.05	1.0S	1.06	0.92	1.19	1.06	5.30
Yeast extract	0.94	0.90	0.94	0.78	1.02	0.916	4.5S
Mean.....	1.095	1.07	1.025	0.952	1.115		21.03
T_j.....	4.3S	4.2S	4.10	3.81	4.46		

Total sum of squares

$$1.21^2 + 1.18^2 + \cdots + 1.19^2 + 1.02^2 - 21.03^2/20 = 0.234$$

Sum of squares between means of species

$$\frac{4.38^2 + 4.28^2 + 4.10^2 + 4.46^2 + 3.81^2}{4} - 21.03^2/20 = 0.067$$

Sum of squares between means of nitrogen source

$$\frac{5.54^2 + 5.61^2 + 5.30^2 + 5.48^2}{5} - 21.03^2/20 = 0.133$$

The remainder, $0.234 - 0.067 - 0.133 = 0.34$, is due to residual variation and will be used for estimate of experimental error. This use assumes that any contribution to the residual variation due to interaction of species and source of nitrogen is negligible in comparison with the portion due to error of experiment, so that practically all of the observed variance in this term may be ascribed to random experimental error. This assumption may of course not be true and could easily be tested by replicating the estimations of the respiratory quotients.[26] The analysis of variance is now easily set up:

VARIATION	D.F.	SUM OF SQUARES	MEAN SQUARE	F	1% POINT
Species...............................	4	0.067	0.0168	5.94	5.41
Nitrogen source......................	3	0.133	0.0443	15.65	5.95
Residual.	12	0.034	0.00283		

It is evident that the value of F for both species of organism and source of nitrogen are much larger than would be expected to occur by random sampling

[26] Thorne, Neal, and Walker (private communication) did use replicates, but the complete data are not included in their paper. For purposes of illustration we have used the data actually given there.

even once in 100 times. We conclude then that our original null hypothesis, that the data may be regarded as independent samples from the same normal population (in particular, that the differences in species and nitrogen source had no effect), is incorrect. Accordingly, we accept the alternative with regard to which the test is most powerful, namely, that the differences in species and in nitrogen source did affect the outcome. We may want to know now which of the treatment means differ from the others. As shown in the table, the estimated variance of a single determination is 0.00283, so that a difference between an arbitrary pair of means of *sources of nitrogen* would be tested by

TABLE 8

Logarithms of milligrams nitrogen fixed by varieties of Medicago sativa inoculated with strains of Rhizobium meliloti

STRAIN OF RHIZOBIUM	VARIETIES OF MEDICAGO SATIVA					
	Hairy Peruvian		Ladak		Grimm	
	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
101	0.92	1.53	0.36	0.83	1.38	1.43
	0.97	1.55	0.32	0.86	1.28	1.53
105	1.09	1.40	1.01	1.37	1.40	1.58
	0.94	1.51	0.74	1.27	1.34	1.42
107	1.34	1.48	1.27	1.48	1.32	1.49
	1.44	1.52	1.10	1.22	1.45	1.46
111	0.94	1.42	1.06	1.60	1.37	1.34
	1.22	1.12	1.04	1.56	1.43	1.34
115	1.02	1.39	1.25	1.18	1.19	1.29
	1.06	1.12	1.18	1.21	1.35	1.29
129	1.35	1.43	1.30	1.39	0.83	1.44
	1.41	1.61	1.11	1.30	0.73	1.54

dividing by an estimated standard error of $\sqrt{0.00283(2/5)} = 0.0336$ to get a t with 12 degrees of freedom. The 5% point for t with 12 degrees of freedom is 2.18, hence a difference of $(2.18)(0.0336) = 0.073$ between an arbitrary pair of *source* means may be regarded as significant at the 5 per cent level. Similarly, $(2.18)(0.0377) = 0.082$ constitutes the 5 per cent level of significance for differences between an arbitrary pair of *species* means.

Certain reservations about the use of such comparisons should be noted. When the difference between a particular pair of means is compared with the corresponding 'minimal significant difference', as the foregoing calculated differences are often called, the interpretation of the outcome depends upon whether the decision to compare these particular means was reached before or after an examination of the data. If before, then, when the observed difference exceeds the minimal significant difference, it may be regarded as indicating a

real, underlying difference. If after, then a difference exceeding the minimal significant difference should be regarded as merely pointing to a comparison which may warrant special attention in further research. Also, observe that among m means only $(m - 1)$ independent comparisons can be made.

The final example to be considered deals with a more complex type of analysis and illustrates the importance of the interaction terms. Burton and Wilson (14) investigated in greenhouse trials the nitrogen-fixing ability of three varieties of *Medicago sativa* L. when inoculated with six strains of *R. meliloti*. The experiments were repeated during different seasons of the year to determine whether this factor affected the results. The logarithms of the quantity of nitrogen fixed per pot of 10 plants for two experiments are summarized in table 8. Only representative calculations which show how the variances are estimated will be given. From table 8 the total sum of squares, $\Sigma(X - \bar{X})^2 = 5.0429$, is determined as has been already illustrated. Next, a new table is made similar to table 8 but in which the duplicate samples have been combined. The items in this second table will be referred to as X_2 ; we calculate the total sum of squares for this table by the following:

[21]
$$\frac{\Sigma X_2^2}{2} - \frac{T^2}{N}$$

where Σ denotes the summation over all values of X_2, and $T = \Sigma X_2 = $ sum of all original observations. Note that the correction term, the square of the total of a table divided by N, will be the same for all steps in a given analysis. The sum of squares obtained by formula 21 is 4.7039, and the difference between this and the original total sum of squares, $5.0429 - 4.7039 = 0.3390$ is that due to 'error', since by combining the duplicates we have eliminated the variation due to this source. This error sum of squares will have 36 degrees of freedom because each of the 36 pairs of duplicates will contribute 1 degree of freedom. We now make another table in which the effect of experiment is disregarded by adding together corresponding items from the two experiments:

STRAIN OF BACTERIA	VARIETY OF HOST PLANT			
	Hairy Peruvian	Ladak	Grimm	T_i
101	4.97	2.37	5.62	12.96
105	4.94	4.39	5.74	15.07
107	5.78	5.07	5.72	16.57
111	4.70	5.26	5.48	15.44
115	4.59	4.82	5.12	14.53
129	5.80	5.10	4.54	15.44
T_j	30.78	27.01	32.22	90.01

Total sum of squares in this table is

$$\frac{\Sigma X_4^2}{4} - \frac{T^2}{72} = \frac{4.97^2 + 4.94^2 \cdots 5.12^2 + 4.54^2}{4} - \frac{90.01^2}{72} = 2.7054$$

in which X_4 indicates that each term is composed of 4 corresponding items of the original data and Σ here denotes the summation over all values of X_4. The total sum of squares in this table is made of three factors: *Strain of organism* $= \sum_{i=1}^{6} T_i^2/12 - T^2/72 = 0.6033$ with 5 degrees of freedom; *Variety of plant* $= \sum_{i=1}^{3} T_i^2/24 - T^2/72 = 0.6032$ with 2 degrees of freedom; and *Interaction* of these two factors, $V \times S = 2.7054 - 0.6033 - 0.6032 = 1.4989$. The interaction term will have 10 degrees of freedom (5×2), as can be verified by the fact that this table has a total of 17 degrees of freedom and 7 of these are used by the simple factors. To obtain the effect of *Experiment* and its interaction with variety of plant, another table is constructed in which the items are classified according to these two categories as:

	HAIRY PERUVIAN	LADAK	GRIMM	TOTAL
Experiment I...........................	13.70	11.74	15.07	40.51 (T_1)
Experiment II.............	17.08	15.27	17.15	49.50 (T_2)
Difference...........................	3.38	3.53	2.08	8.99

The total sum of squares of this table is composed of: that due to *Variety* (which has been already determined), that due to *Experiment*, and that due to *Interaction* of variety and experiment. These could be calculated in the usual way, but for tables of this type ($2 \times n$), a more rapid method is:

Sum of squares due to experiment

[22]
$$(T_2 - T_1)^2/2N' = 8.99^2/72 = 1.1225$$

with one degree of freedom, where T_1 and T_2 denote the totals for Experiments I and II respectively, and where N' is the number of items represented by each total, in this case $N' = N/2$, where N is the total number of observations in table 8.

Sum of squares due to interaction

[23]
$$\frac{\Sigma d^2}{2k'} - \frac{(T_2 - T_1)^2}{2N'} = \frac{3.38^2 + 3.53^2 + 2.08^2}{24} - 1.1225 = 0.0530$$

where d is the difference between the total for a given variety in Experiment II and in Experiment I, each such total being the sum of k' original observations, and Σ denotes summation over varieties. The entire table has 5 degrees of freedom, of which 2 belong to *Variety*, 1 to *Experiment*, and 2 to their *Interaction*.

A similar table is constructed in which the categories are *Strain of organism* and *Experiment*. The interaction term, *Strain* $\times$ *Experiment* is determined in exactly the same manner, giving 0.2735 with 5 degrees of freedom. The

sums of squares for the several factors and their first order interactions are now added and this sum, 4.3053, taken from the total sum of squares for the X_2 table, 4.7039. The difference, 0.3986, is the sum of squares belonging to the triple (second order) interaction, *Variety* $\times$ *Strain* $\times$ *Experiment*. This has 10 degrees of freedom ($5 \times 2 \times 1$), since the total for all treatments is 35, and 25 of these have been used by the simple factors and first order interactions. The analysis of variance can now be set out as follows:

VARIATION	D.F	SUM OF SQUARES	MEAN SQUARE	F	5% POINT	1% POINT
Variety	2	0.6032	0.3016	32.02	3.27	5.26
Strain	5	0.6033	0.1207	12.81	2.48	3.59
Experiment.. ...	1	1.1225	1.1225	119.16	4.12	7.41
V $\times$ E	2	0.0530	0.0265	2.81	3.27	5.26
S $\times$ E	5	0.2735	0.0547	5.81	2.48	3.59
V $\times$ S	10	1.4989	0.1499	15.91	2.13	2.90
V $\times$ S $\times$ E.	10	0.5495	0.0550	5.84	2.13	2.90
Error	36	0.3390	0.00942			
Total	71	5.0429				

The interpretation should give no difficulty. The values of F corresponding to the variance due to the single factors, *Variety* and *Strain*, exceed the 1% point indicating that at least one variety of the host plant was superior to the others independent of the strain of bacteria used as the inoculum, and that at least one strain of the bacteria was better than the others independent of the host plant. Obviously the experiment factor was significant since the design was such that more nitrogen would be fixed in one experiment than the other. Considering the first order interaction terms, it appears that the varieties responded identically in the two experiments, but the strains did not. The important interaction term is the *Variety* $\times$ *Strain*; the high value of F corresponding to it shows that the efficiency of a strain in fixing nitrogen varies with the host plant used—thus establishing "host plant specificity" for this plant-bacterial group (14). Of interest is that this interaction of variety and strain varied with the experiment as shown by the significant F value for the second order interaction term, V $\times$ S $\times$ E. The possible significance of this point (verified in further experiments) for symbiotic nitrogen fixation is discussed in the original paper. Once significance for a factor is established, some indication of the particular bacterial strains and varieties of host plant responsible can be obtained by calculating the appropriate error from the error of the single sample, 0.00942, and testing *means* or *totals* by the usual t formula.

This example illustrates several important aspects of the analysis of variance which should be considered in somewhat more detail before finishing our discussion.

1. *The equality of variances within treatments.* The requirement that all the observations should be of equal precision, *i.e.*, have the same standard devia-

tion, needs emphasis as it is sometimes ignored. Cochran (21) stresses the importance of this requirement and illustrates by examples how a mechanical application of analysis of variance to data of varying precision can lead to absurd conclusions. In the present experiment, the treatments, including time of experiment, caused a wide variation in the nitrogen content of the plants. The difference in nitrogen content, of course, is merely another way of stating that the plants differed in size. *A priori*, one would not suppose that *variation* among a population of plants of one size would equal that of another population in which the plants were 2 to 3 times as large; experience with plants under the conditions used in this experiment has verified this conclusion. Since under these conditions the growth of the plants is approximately logarithmic, it seems reasonable to assume that if the original data, total nitrogen in 10 plants, are transformed by taking the logarithm, the variances of the transformed data would not greatly differ among the different treatments. The logical transformation is not always so evident as in this case (53). Thus, percentage should be transformed to an angle, $\theta = \arcsin \sqrt{p}$, where p denotes an observed *proportion*. Employed originally by Fisher (29), the application of this transformation to experimental data has been promoted by Zubin (125), Bliss (5, 6), and Clark and Leonard (16). Of special importance to bacteriologists is the square root transformation advanced by Bartlett (1, 2) for use in connection with variables having Poisson distributions. If suspicion exists that the variances are unequal, some function of the observations in which equal variances might be expected should be taken before proceeding with the analysis. Bartlett (2) has shown how to test a set of variance estimates for homogeneity.

2. *Design of experiment.* It is essential that the various treatments have an equal chance of being exposed to those factors in the experiment which are not under control. This is a matter of *design of experiment*. Little more can be noted here than some of the methods available for insuring proper design for statistical treatment of the data; details are given in the monograph of Fisher (33) as well as in any modern statistical text which deals with agricultural experimentation. In the nitrogen fixation studies, only 36 pots were used in each experiment, as this number could be readily placed in a rather small space on the greenhouse bench. The position of a given pot in the area used was determined by chance (drawing of a card or use of a table of random numbers). Had the experiment required more pots, *e.g.*, 4 replicates instead of 2, so that the space occupied in the greenhouse would be rather extensive, the introduction of blocks would be advisable. The greenhouse bench would be divided into 4 blocks in such a way that the conditions (light, temperature, drafts) in a given block would be reasonably constant, then one pot of each particular treatment placed within each block, its exact position being determined by chance as before. In this case the replicates of a particular treatment are not interchangeable, but are grouped with the corresponding replicate of the other treatments in the same block. Thus the block becomes one of the 'treatments'. The sum of squares due to this factor is removed from the total in the statistical analysis,

which prevents the error variance from increasing because of differences in the environment of the different blocks.

This technique is extremely important in field trials in which soil heterogeneity is one of the chief sources of variance. By proper experimental design, the variance due to differences in soil fertility in different parts of the experimental plot can be estimated and allowed for in the analysis. This increases the permissible number of replicates, but imposes a limit on the number of treatments, namely that which can be placed within the relatively homogeneous block. An interesting extension of thus correcting for soil heterogeneity is the Latin square which can be used if the experiment is designed so that the number of replicates equals the number of treatments. As can be seen in the following example of a four-fold Latin square in which A, B, C, and D represent treat-

B	A	D	C
A	D	C	B
C	B	A	D
D	C	B	A

ments, a particular treatment appears exactly once in each row and column. In the analysis, sums of squares due to differences between rows and to differences between columns are segregated thus making allowance for gradients of soil fertility, etc., in two directions within the field. Although the Latin square has been used primarily in field plot trials, obvious applications suggest themselves in other research areas, e.g., in bacteriology, arrangement of cultures in an incubator so as to be able to make allowances for temperature gradient.

Another point concerned with experimental design illustrated by the final example deals with the estimate of the error. If single samples had been taken as was done in the preceding example, instead of duplicates, the row labelled *Error* in the analysis of variance table would be absent, and the V × S × E interaction would have to serve as the best available estimate of the experimental error. If this had been done, the mean squares due to strain differences and to interaction of strain with experiment would have been judged 'non-significant'. Although perhaps of secondary interest in this particular experiment, the resulting difference in interpretation emphasizes the desirability of replication and the danger of presuming that a certain interaction is non-existent in order to use the corresponding mean square as an estimate of experimental error.

3. *The error variance.* An analysis of variance will not generally be needed to evaluate the relative merits of treatments differing widely in effectiveness, and, indeed, will be unavailable in such cases if the observations corresponding to the respective treatments differ in precision. The refinements of analysis of

variance are needed principally when the differences between the treatments are slight, and, fortunately, in such cases the observations are generally of approximately equal precision so that the analysis of variance technique *is* available. When treatment differences are not great, efficient analysis will aid mateirally in this detection. Furthermore, this analysis of variance provides additional advantages. First, we obtain an estimate of experimental error, for comparing any two treatment means, which is based on all the observations in the experiment rather than one based just on the observations corresponding to the treatments whose means are being compared. Hence, the test of significance has greater power, *i.e.*, has a larger chance of detecting any real difference between the treatments. Second, factors which are known to affect the result, and which in classical experimentation are kept constant in order to make possible a determination of experimental error, may be varied within the limits imposed by the design employed and allowed for in the analysis, thereby giving reality to an experiment which otherwise might suffer from idealization possible only in the laboratory.

To illustrate these points, let us consider an experiment in which four media are to be compared for their ability to bring about some desired growth response in the commercial production of yeast. Suppose that laboratory facilities would allow 16 determinations to be made with each medium. If all media were kept in some carefully controlled laboratory environment, then the analysis of variance would be:

Test of 4 media in one environment

Variation	Degrees of Freedom
Between media........	3
Within media (error)	60

The difference between any two media will here be compared with an estimate of experimental error based on 60 degrees of freedom, whereas a pair-wise comparison using only the data for the media compared would employ an estimate of error based on 30 degrees of freedom. Since such a large number of replications are involved, the increase in precision by use of analysis of variance is slight, as can be seen from the fact that the 5 per cent level of t for 30 degrees of freedom is 2.04, and for 60 degrees of freedom is 2.00. Using the pair-wise comparison as a standard, it is seen that by planning to use analysis of variance, the number of determinations to be made on each medium could be reduced to 8 to get comparable accuracy, since the 5 per cent significance level of t for $4 \times 7 = 28$ degrees of freedom is 2.05.

It would be impractical, however, to make such an experiment in which the media were kept in a carefully controlled laboratory environment, since it is well established that the relative suitability of a medium for an organism varies with factors in the environment such as temperature, aeration, size of inoculum. Suppose each of these factors was introduced into the experiment at two levels, then duplicates of each treatment would be possible in the 64 cultures since $2(4 \times 2 \times 2 \times 2) = 64$. The analysis of variance would then be:

Test of 4 media in different environments

Variations	Degrees of Freedom	Variations	Degrees of Freedom
Main Effects:		*2nd Order Interactions:*	
Between media.................	3	M × T × A.....................	3
Between temperatures..........	1	M × T × I.....................	3
Between aerations.............	1	M × A × I.....................	3
Between inocula...............	1	T × A × I.....................	1
1st Order Interactions:		*3rd Order Interaction:*	
M × T........................	3	M × T × A × I...............	1
M × A........................	3		
M × I........................	3	*Between Duplicates (Error)*........	32
T × A........................	1		
T × I........................	1		
A × I........................	1		

The *actual* experimental error will be no larger in the complex experiment, but its estimate, being based on 32 degrees of freedom instead of 60, will have lost some precision, but it is as precisely determined as would have been the case in a pair-wise analysis with 16 determinations for each medium in a single environment. This loss, however, is more than compensated for by the greatly increased information obtained concerning the influence of temperature, aeration, and size of inoculum on the response of yeast in different media together with the various interactions. The paper of Brandt (9) may be consulted for the working of an actual problem such as the one outlined.

An interesting example of the employment of analysis of variance in the statistical control of a laboratory technique is given by James and Sutherland (55, 56, 57) in their studies on the accuracy of the plate count in enumerating soil microörganisms. They investigated a number of factors which might be expected to affect the counts, such as aliquot of soil taken, method of dilution, of pouring plates, and of incubating. Their analysis indicated that both aliquot taken and dilution[27] were important in affecting the count, but the other details of technique investigated had less influence. Though they did not feel justified in making definite recommendations on the basis of their findings, they suggested that if one is limited to a certain number of plates, a more accurate estimate will be obtained if the number of aliquots and dilutions is increased at the expense of replication of a single dilution. The fact that different dilutions frequently gave rise to different estimates was considered, and a method for correcting the estimates so as to be interchangeable was suggested (56).

REGRESSION AND CORRELATION

A primary function of all research is to determine *relationships* between two or more quantities. To know *how* phenomena are related is essential to all scien-

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[27] To avoid confusion in our discussion of the statistical control of plate counts, we have disregarded errors arising from technique as contrasted with the sampling error. In actual practice, however, it is recognized that errors in dilution, *etc.*, may become just as important in affecting the reliability of a count as the other. Jennison and Wadsworth (58) discuss this aspect of variation and have furnished a table for correction of dilution errors for various deviations in pipette and dilution blanks.

tists from the theorist who integrates the relationships into hypotheses regarding the *why* of nature to the technician who wants to know the value of one quantity from observations on another. Probably the most obvious way to judge the relationship between two variables[28] is by plotting the values on graph paper and noting the trend. Usually an effort is made to obtain a linear relationship as this simplifies interpretation and use. If the original data do not yield a straight line, its trend often suggests the proper function, and by suitable transformations a linear relationship can be secured. Having plotted the data so that they appear to be linear, the investigator may merely draw in the line which to his eye appears to fit them. Although this is probably not objectionable if the fit of the observations to the line is very close, it introduces an undesirable subjective element and an opportunity for bias. Sometimes the fit is fictitiously good, because of the scale used for plotting (37), and disagreement regarding interpretation ensues.

Statistical theory points out that the criterion of goodness of fit which should be adopted in a given instance depends upon the nature of the random variation affecting the variables, and provides objective methods of fitting which lead to the best fitting line as judged by the appropriate criterion. In biological research, methods for obtaining a relation between two variables which it is hoped will be sufficiently close to the true relationship for the purposes in mind involve the following steps[29]:

(a) It is assumed that the pairs of observational points, $(x_1y_1) \cdots (x_ny_n)$, differ from the 'true' points as a result of biological variation and errors of measurement in either x or y or both. 'True' is used in the sense that for a fixed value of x observed values of y will be randomly distributed about a central value, called the 'true' value, the exact nature of which depends upon the character of the distribution. When the distribution is normal, the *mean* is termed the 'true' value.

(b) From theoretical considerations or from the appearance of the graph *some* mathematical relationship between x and y is assumed. We shall restrict ourselves to consideration of the linear type:

$Y = \alpha + \beta x$ and $X = \gamma + \delta y$, in which the manner of conducting the experiment usually determines which is to be the independent variable (26).

(c) Estimates of the constants, *e.g.*, a and b for α and β, are chosen which will make the resulting line 'best' fit the observations. When the random variation is normal with the same standard deviation throughout the range of x and y considered, the fit is 'best' when the sum of the squares of the deviations of the observations from the line chosen is a minimum. We shall restrict the present discussion to the case where normal random variation is manifest in the observed

[28] In this paper attention will be confined to simple regression coefficients since the extension of the methods to problems involving 3 or more variables can be found in any of the standard references given in the bibliography.
[29] A fuller discussion of the concepts and principles involved has been given by Eisenhart (26).

values of y, but the values of x are exactly determined, so that an observed point can deviate from the 'true' point in the y direction only.

(d) Tests of significance are then carried out to determine how good the fit actually is; the outcome of these will be the basis for deciding whether the chosen function can adequately describe the actual relation between the x's and y's.

The methods for carrying out these steps can best be followed by working through a specific example. Table 9 provides data on the fixation of nitrogen by inoculated red clover plants kept in an atmosphere containing H_2. When

TABLE 9

Fixation of nitrogen by inoculated clover plants in presence of H_2*

ARRAY	TIME IN DAYS (x)	LOG MG N FIXED (y)	SUM OF y ARRAYS (T_i)
1	0	0.520 0.546 0.612	1.678
2	17	0.843 0.844 0.835	2.522
3	31	1.090 1.189 1.199	3.478
4	50	1.484 1.559 1.496	4.539
T_x.	294		
T_y.			12.217

* $pN_2 = 0.15$; $pO_2 = 0.2$; $pH_2 = 0.15$; $pHe = 0.5$ atm.; $n_i = 3$ in all arrays.

the original data were plotted, a logarithmic function was suggested which was verified by plotting *log mg N* against *time in days*. It should be noted that in fitting these data it is clear that *time in days* constitutes the independent variable, since its values are determined by the will of the experimenter, and, provided the latter can count accurately, its values are not subject to experimental error. The postulated relationship is:

[24] $$Y = \alpha + \beta X$$

where Y = mg N fixed, X = time in days.
The constants in this equation are estimated by:

[25] $$a = \bar{y} - b\bar{x}, \text{ and}$$

[26]
$$b = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\Sigma(x - \bar{x})^2} = \frac{\Sigma x(y - \bar{y})}{\Sigma(x - \bar{x})^2}$$

where $\bar{x}$ and $\bar{y}$ denote the mean values of the 'observed' values of x and y, respectively, and Σ denotes summation of all pairs of observations. In actual calculation, use is made of the following identity where N = number of pairs of observations:

[27] $\Sigma(x - \bar{x})(y - \bar{y}) = \Sigma xy - (\Sigma x)(\Sigma y)/N,$

$= 0(0.520 + 0.546 + 0.612) + 17(0.843 + 0.844 + 0.835) \cdots$ etc.
$- (294)(12.217)/12 = 377.642 - 299.316 = 78.326$

From equation [15],

$\Sigma(x - \bar{x})^2 = 3(0^2 + 17^2 + 31^2 + 50^2) - 294^2/12 = 11250 - 7203 = 4047$

hence, $b = 78.326/4047 = 0.01935$

$a = \bar{y} - b\bar{x} = 12.217/12 - 0.01935(294/12) = 0.544$, and the 'best' line is:
$Y = 0.544 + 0.01935X.$

For testing the significance of these constants, the total sum of squares $\Sigma(y - \bar{y})^2$, must be divided into its several components appropriate to the test, and an analysis of variance made. By equation 15, $\Sigma(y - \bar{y})^2 = 0.520^2 + 0.546^2 + \cdots 1.559^2 + 1.496^2 - 12.217^2/12 = 1.53552$. For testing the significance of regression, which means testing whether b is significantly different from zero, $\Sigma(y - \bar{y})^2$ is divided into two parts: first, that due to regression, *i.e.* deviations of points on the regression line from the mean of the y's, which equals

[28] $\Sigma(Y - \bar{y})^2 = b^2\Sigma(x - \bar{x})^2 = (0.01935)^2(4047) = 1.5153,$

and the remainder which accounts for the deviations of the observed points from the regression function, $\Sigma(y - Y)^2 = 1.5355 - 1.5153 = 0.0202.$

The objection may well be raised that testing for regression in this example is unnecessary and somewhat artificial, since it is obvious from mere inspection of the raw data that the quantity of nitrogen fixed increases with time. Although it is true that in this particular instance it is superfluous to test whether b differs from zero, this is not always so, and indeed many times, it is the most significant test made. Hence, in order to illustrate the method and also, because some of the values will be needed in the test for linearity, we include this step. The analysis is:

VARIATION	SUM OF SQUARES	D.F.	MEAN SQUARE	F	5% POINT
Regression.. . .	1.5153	1	1.5153	750.1	4.96
Deviations from regression . .	0.0202	10	0.00202		

As would be expected F is well beyond the 5% point.

Although the foregoing analysis confirms the existence of regression as measured by a linear function, it does not indicate the adequacy of a straight line to represent the actual relationship. To test this, we divide the total sum of squares into two parts, one of which represents that portion due to the means

of the arrays differing from $\bar{y}$, (and therefore to differences between the arrays) and the other, that portion due to the scatter of values within the several arrays.

[29]
$$\Sigma(y - \bar{y})^2 = \Sigma n_i(\bar{y}_i - \bar{y})^2 + \Sigma\Sigma(y - \bar{y}_i)^2$$

$$\text{Total} \qquad \text{Between} \qquad \text{Within}$$
$$\text{arrays} \qquad \text{arrays}$$

The total degrees of freedom is $(n - 1)$ of which $(q - 1)$ belongs to the *Between Arrays* and $(n - q)$ to the *Within Arrays*. In the foregoing equation n_i denotes the number of samples in the ith array with mean $\bar{y}_i$, with $\Sigma n_i = n$, and with q number of arrays. The double summation signs indicate that the sum of squares for the deviations within arrays is to be summed for all q arrays. The sum of squares due to differences *Between Arrays* is further broken up into a part due to deviations of means of arrays from the regression line with $(q - 2)$ d.f. and a part due to the regression itself (1 d.f.):

[30]
$$\Sigma n_i(\bar{y}_i - \bar{y})^2 = \Sigma n_i(\bar{y}_i - Y)^2 + b^2\Sigma(x - \bar{x})^2$$

The linearity of regression is tested by comparing the mean square corresponding to deviations of means of arrays from the regression line with the mean square corresponding to *Within Arrays*. Since

[31]
$$\Sigma n_i(\bar{y}_i - \bar{y})^2 = \Sigma T_i^2/n_i - T_y^2/N$$
$$= \frac{1.678^2 + 2.522^2 + 3.478^2 + 4.539^2}{3} - \frac{12.217^2}{12} = 1.5205$$

then $\Sigma n_i(\bar{y}_i - Y)^2 = 1.5205 - 1.5153 = 0.0052$
and $\Sigma\Sigma(y - \bar{y}_i)^2 = 1.5355 - 1.5205 = 0.0150$.
The analysis of variance accordingly is:

VARIATION	SUM OF SQUARES	D.F.	MEAN SQUARE	F	5% POINT
Deviations of means of arrays from line.	0.0052	2	0.0026	1.37	4.46
Within arrays..................... ..	0.0150	8	0.0019		

As the value of F is definitely less than that of the 5 per cent point, it is concluded there is no evidence of departure from linearity.

When a value of b has been determined in each of two independent experiments, an extension of the t test can be used to determine whether the two values of b differ significantly:

[32]
$$t = \frac{(b_1 - b_2)}{\left[\frac{(N_1 - 2)s_1^2 + (N_2 - 2)s_2^2}{N_1 + N_2 - 4} \{1/\Sigma(x_1 - \bar{x}_1)^2 + 1/\Sigma(x_2 - \bar{x}_2)^2\} \right]^{\frac{1}{2}}}$$

in which

[33] $$(N - 2)s^2 = \Sigma(y - \bar{y})^2 - b^2\Sigma(x - \bar{x})^2$$

and t has $(N_1 + N_2 - 4)$ d.f. This formula should be used only when s_1^2 and s_2^2 do not differ significantly, otherwise the u or v tests of Welch (105) are available. The necessary data for testing estimates of a and b obtained in some experiments with clover plants grown in atmospheres with different partial pressures of H_2 follow:

pH_2	a	b	N	$(N-2)s^2$	$\Sigma(x-\bar{x})^2$	$\bar{x}$
0.15	0.544	0.01935	12	0.0202	4047	24.50
0.35	0.565	0.01606	9	0.0171	3231	21.77

The first step is to compare the variances by calculating $F = (0.0171/7)/(0.0202/10) = 1.21$, which is well below the 5 per cent point of 3.15 for 7 and 10 d.f. As the variances appear to be homogeneous, the t test may be applied:

$$t = \frac{0.01935 - 0.01606}{\left[\dfrac{0.0202 + 0.0171}{12 + 9 - 4}\left\{\dfrac{1}{4047} + \dfrac{1}{3231}\right\}\right]^{\frac{1}{2}}} = 3.21 \text{ with 17 d.f.}$$

Since this value of t exceeds the 1 per cent point, it appears that the observed difference in the b's is significant.

The difference between the a's may be tested by means of the following formula when s_1^2 and s_2^2 do not differ significantly.

$$t = \frac{a_1 - a_2}{\left[\dfrac{(N_1 - 2)s_1^2 + (N_2 - 2)s_2^2}{N_1 + N_2 - 4}\left\{\dfrac{1}{N_1 - 2} + \dfrac{\bar{x}_1^2}{\Sigma(x_1 - \bar{x}_1)^2} + \dfrac{1}{N_2 - 2} + \dfrac{\bar{x}_2^2}{\Sigma(x_2 - \bar{x}_2)^2}\right\}\right]^{\frac{1}{2}}}$$

[34]

$$= \frac{0.565 - 0.544}{\left[\dfrac{0.0171 + 0.0202}{9 + 12 - 4}\left\{\dfrac{1}{7} + \dfrac{21.77^2}{3231} + \dfrac{1}{10} + \dfrac{24.50^2}{4047}\right\}\right]^{\frac{1}{2}}} = 0.7$$

The difference between the a's is clearly not significant, which means, of course, that log mg. nitrogen at $t = 0$ was the same in each series, i.e., the two tests started together.

Regression statistics have numerous applications in bacteriology and allied fields several of which will be considered briefly.

Calculation of k values. In bacteriological research it is often advantageous to use the *rate* of growth (rate of respiration, rate of nitrogen fixation, *etc.*) rather than *total* growth (total respiration, total nitrogen fixed, *etc.*). Since these functions in many instances increase logarithmically with time, the traditional measure of growth rate is the k value defined: $k = (1/t) \ln [(a + y)/a]$ in which a represents the growth at $t = 0$, and y the increase after time t. If log $(a + y)$ is plotted against t, the slope of the resulting line multiplied by 2.303 will estimate k. Thus, in the example just discussed, the specific rate constant of nitro-

gen fixation for a pH_2 of 0.15 atm. is: $0.01935 \times 2.303 = 0.0445$. Not only can the best estimate of k be made but also the significance of observed differences in k values can be determined by testing the b's from which they were derived. The papers of Wilson and his associates furnish several examples of this use of regression coefficients (60, 113, 116, 120, 121).

Estimation of k values by this method is greatly facilitated if experiments are planned so that the calculations are reduced. For example, nitrogen fixation by *Azotobacter* can be estimated indirectly in a Warburg microrespirometer by measuring the increase in rate of respiration with time. For routine determinations in our laboratory, a standard method has been adopted in which five readings are taken hourly. Under these conditions, both Σx and $\Sigma(x - \bar{x})^2$ equal 10; N is 5 and $\bar{x}$ is 2. Reference to formulae 25 and 26 shows that both a and b can be rapidly calculated since only Σy and Σxy must be evaluated, the other terms being determined mentally. Calculation of the error in b requires somewhat more effort, but if a calculating machine is available, all the statistics can be determined *without bias* as rapidly as b is estimated by the usual graphical procedure.

After a period of time, it may not be necessary to determine the error variance, s^2, except for a check. We have calculated variances from over 100 trials and shown them to be 'homogeneous', *i.e.*, all belong to the same population, so that their mean, $\bar{s}^2$, provides a reliable estimate of the expected variance under the prescribed conditions.[30] From $\bar{s}^2$ and $\Sigma(x - \bar{x})^2 = 10$, it was determined that two k's must differ by 13 to 17 per cent in order to be significant. Thus in routine work under the 'standard' conditions, only the b's are determined; from these the k's are calculated and compared. Occasionally, the variance, s^2, is estimated to make certain that the method is under statistical control. If significantly excessive (or deficient) variances are obtained, the technique is examined for possible interfering factors. Not only does this serve as a red light for trouble, but it provides an objective test for the learning process in new students. Before he starts his research, a beginner makes several practice runs; he continues these until the error in his b's is commensurate with that established by competent workers.

Test of a hypothesis. Frequently a hypothesis under test dictates the method of plotting the data and usually by mathematical manipulation the suggested function can be put in linear form. This is illustrated by data from Wilson, Burris and Lind (115) in figure 6. The hypothesis is that the initial steps in nitrogen fixation by *Azotobacter* can be represented by the enzyme mechanism first formulated by Michaelis and Menten (27). Lineweaver and Burk (61) showed that if the hypothesis is correct a straight line should result when the reciprocal of the rate of fixation $(1/k)$ is plotted against the reciprocal of the partial pressure of nitrogen $(1/pN_2)$. Likewise, if at a given pN_2 the velocity *relative* to the maximum velocity is k^*, then pN_2/k^* should be a linear function of pN_2. The figure shows that straight lines were obtained by both methods of plotting. The fit of the points to the line at the left does not appear to be so good, but this results primarily from the scale used. Statistical tests demonstrated that a straight line was satisfactory in both instances.

Another test of this hypothesis based on regression statistics was also described in the same paper (115). If the hypothesis holds, the rate of fixation should be less at a pN_2 of 0.2 atm. than at 0.8 atm. Calculation showed that the

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[30] The test for homogeneity among several estimated variances constitutes another application of the χ^2 distribution (see p. 122 ff.). The details of this test can be obtained in a textbook on statistics, *e.g.*, Rider's (79, p. 102), or from the original (2).

error in k would mask the expected difference in an *individual* experiment. But, by combining the results from a number of experiments the expected difference was demonstrated. Moreover, by increasing the number of observations in a single trial, the error was decreased so that significant differences were detected in the individual experiments. Wilson and his collaborators (116) have also used regression statistics for determining whether an inhibitor of an enzyme system acts competitively or non-competitively.

Estimation of μ values. In enzyme studies, definition of the physical-chemical characteristics of the system investigated is frequently useful—for example, to determine if the enzyme system in one organism is reasonably similar to a corre-
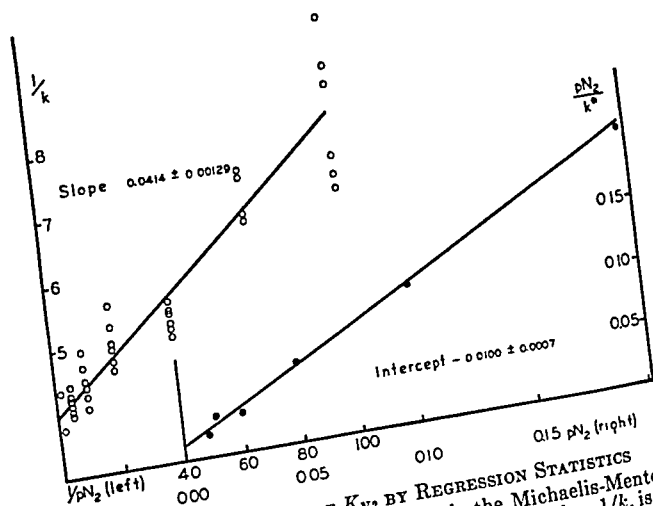
FIG. 6. ESTIMATION OF K_{N2} BY REGRESSION STATISTICS

On the hypothesis that the enzyme mechanism is the Michaelis-Menten type (27), a straight line should result when the reciprocal of the rate of reaction, $1/k$, is plotted against the reciprocal of the substrate concentration, $1/pN_2$ (left). Likewise, if pN_2 is divided by the rate of reaction relative to the maximum rate, under the conditions of the experiment, and this value, pN_2/k^*, is plotted against pN_2, a linear relationship obtains (right). Data are from Wilson, Burris and Lind (115).

sponding system in another (27, chap. X). One important characteristic is the response of the system to changes in temperature. The theory of chemical kinetics suggests that if the log of the rate of reaction is plotted against the reciprocal of the absolute temperature, a straight line should result whose slope measures the 'energy of activation' of the compound undergoing reaction. In chemistry this is called E, but in biology it is usually denoted by μ to signify that it may not be necessarily identified with an energy of activation but may represent a complex of factors. With some systems, e.g., dehydrogenases, this method of plotting gives quite satisfactory linear relationships, but with more complex reactions, such as respiration, the empirical function suggested by Beleradek (13) is often better. For many purposes it is immaterial whether a theoretical interpretation is placed on these slopes; the important fact is that they can be determined with known precision and can be compared by a method free

of subjective bias.   Examples of this use are described by Tam and Wilson (97), and by Burris and Wilson (13).

Biological assay.[31]   Figure 7 represents a most important type of application of statistics to biological phenomena: the assay of a compound based on the
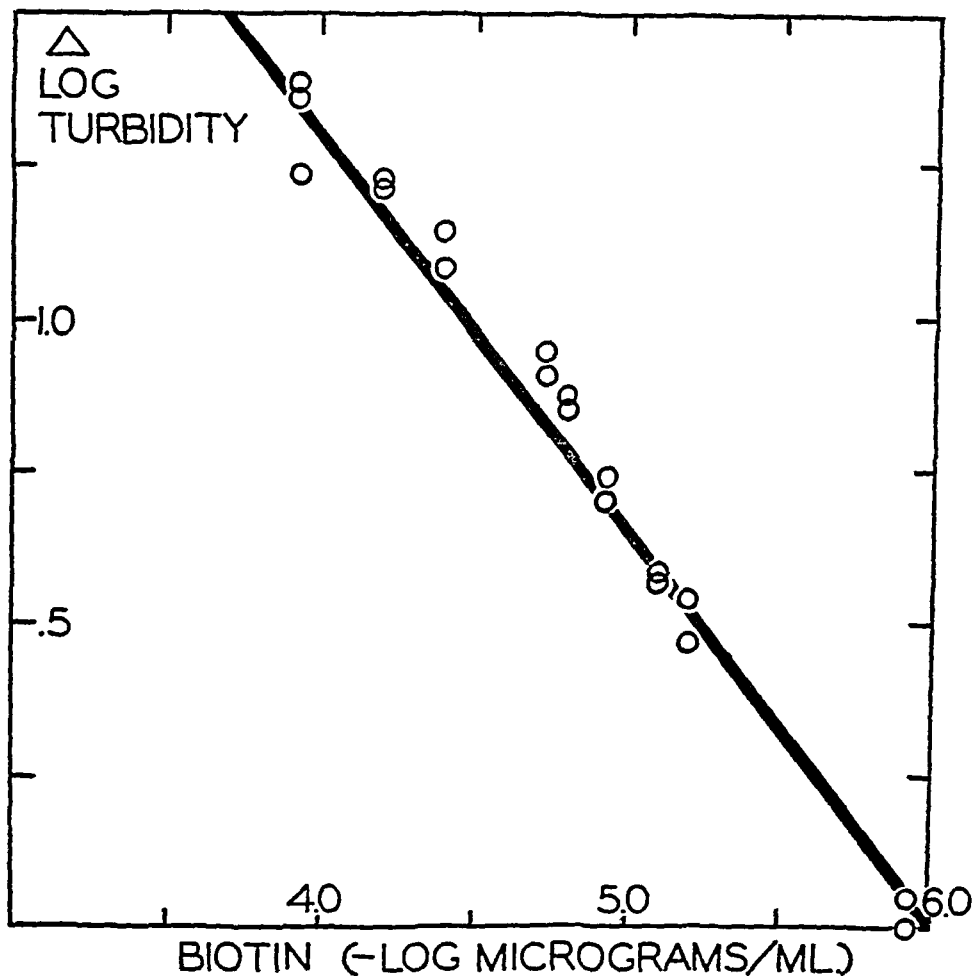
FIG. 7. USE OF REGRESSION STATISTICS IN BIOLOGICAL ASSAY

The graph is a standardization curve relating the growth of *Rhizobium trifolii* to the biotin concentration of the medium.   The growth is estimated by the increase in the turbidity of a supension, measured in a photoelectric colorimeter.   From such a graph, biotin in an unknown could be estimated by observing the effects of different quantities added to the basic medium on the growth of these bacteria.

[31] Space does not permit a thorough discussion of this important field for application of statistics to biological problems.   The outstanding contributions of Bliss (3, 4, 7), Gaddum (38), Wilcoxon and McCallan (111), and others during the past decade has removed much of the sting of Burn's (11) 1930 observation that "Biological assay, as carried out by the majority of the workers in the world, still remains a subject for amusement or despair, rather than for satisfaction and self-respect."   The cited references discuss the special methods developed for experimental design and analysis of the data together with examples of their use, including such fields of bacteriological interest as: toxicity tests on fungicides, response of animals to the administration of therapeutic drugs, bio-assay of antitoxin preparations, *etc.*

response of a biological agent. In this particular case *Rhizobium trifolii* was grown in colonies on a synthetic agar medium to which various levels of a sample of Kögl's biotin had been added; after a suitable period of incubation, the turbidity of six of these colonies suspended in 10 ml of water was determined in an Evelyn photoelectric colorimeter. It was found that under the conditions used in the assay, plotting Δlog turbidity against −(log biotin concentration in μg/ml) gave a straight line. Estimation of this line resulted in the relation:

$$Y = 3.917 - 0.652X$$

in which $X = -$log biotin concentration and $Y = $ Δlog turbidity. In actual experiment, however, the turbidity is determined after a certain quantity of material to be assayed is added to the medium, the biotin concentration being calculated from this reading. For convenience, the equation is solved for X:

$$X = 6.01 - 1.534Y$$

The point to be emphasized is that even though X will be the dependent variable in actual use, the fitting of the line must be done with it as the independent variable, since the experimental design requires this procedure (26).

CORRELATION COEFFICIENT

The correlation coefficient between two quantities measured simultaneously is:

[35] $$r = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\sqrt{\Sigma(x - \bar{x})^2 \Sigma(y - \bar{y})^2}} = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{N\sigma_x \sigma_y}$$

This coefficient is particularly useful for measuring the association between two variables when these can be classed according to quantitative standards. (The treatment is readily extended to more than two variables in multiple correlation.) The statistic, r, is obviously related to the regression coefficient but differs from the latter in that its value is absolute in the sense that it is independent of the units used in the measurements. In cases where the data do not indicate which variable is independent, two regression lines are calculated, one which measures the regression of y on x with slope b_{yx}, and another which measures the regression of x on y with slope, b_{xy}. It can be shown that

[36] $$r^2 = (b_{yx})(b_{xy}),$$

i.e., r is the geometric mean of the regression coefficients. The value of r ranges from $+1$ (complete direct dependence) through 0 (complete independence,[32] to -1 (complete inverse dependence).

The distribution of r itself is very skew in small samples and changes its form

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[32] When r is calculated from a set of values depicting a complete independence of x and y, r will be zero, but when $r = 0$, it does not follow that x and y are completely independent only that they are not linearly dependent. Thus $r = 0$ for a set of points lying on, and equally spaced around, the circle $x^2 + y^2 = 4$. For an elaboration see Rider (79).

rapidly as ρ, the true correlation coefficient of which r is the estimate, changes. For this reason it is difficult to correct for the skewness in estimating the probable range of an observed value of r. However, r can be transformed to a statistic,

[37] $$z = 1/2[\ln(1 + r) - \ln(1 - r)] = (1.15) \log \frac{(1 + r)}{(1 - r)},$$

whose distribution is nearly normal with a standard deviation of $1/\sqrt{(N - 3)}$. It follows then that the significance of an observed r will depend on the number, N, of paired observations. Fisher (30) furnishes a table which gives the values which r must reach for different values of N and for different levels of significance. For significance at the 5 per cent level, r must be at least 0.63 if based on 10 pairs of samples, but only 0.28 if based on 50 pairs. To test for significance between two observed values of r, these are transformed into the corresponding z's, and since the standard deviation of the latter is known, the difference can be tested in the usual way using a table of the normal probability curve.

Although r is a useful statistic for a quantitative measure of association, its use, or rather abuse, by some investigators has led to much nonsense. The source of most of this is the false reasoning that a significant correlation between two quantities reveals a causal relationship. Although the natural scientist has not erred with this piece of faulty logic so frequently as the investigator in the social sciences, the former is by no means guiltless. A measure of the improvement that can be made in the error of estimation through knowledge of the value of a correlated variable is given by $1 - r^2$. This quantity measures the percentage of variance retained; obviously unless r is rather high (order of 0.8) there is little improvement. Since the arithmetic of correlation is very similar to that already illustrated with the regression coefficient, it is unnecessary to discuss this in detail; any standard text on statistics will provide examples of the methods for calculation. The use of the correlation coefficient is so wide-spread that illustrations are probably familiar to all; three typical applications from bacteriological literature will be cited.

Edwards and Rettger (25) found that the maximum growth temperature of 104 strains of bacteria representing 18 species was closely correlated with the minimum temperature of destruction of indophenol (cytochrome) oxidase (0.843), catalase (0.845), and succinodehydrogenase (0.774). Martin (65) surveyed different types of Arizona soils for presence of *Azotobacter* and for their nitrogen-fixing ability, pH, and content of certain salts. He found significant negative correlation between the mg nitrogen fixed per gram of soil and the water-soluble sodium and calcium. Rather unexpectedly, no correlation was noted between nitrogen fixed and phosphate content or pH; sulfate and chloride content were correlated with nitrogen fixation only through their association with calcium and sodium. Recently, Vaughn and Levine (102) determined the correlation between significant characteristics of "Intermediate" cultures of the coliform bacteria and, on the basis of the findings, recognized two species which were allocated to the genus *Escherichia*.

TESTING FOR AGREEMENT BETWEEN OBSERVED AND EXPECTED FREQUENCIES

Often in experimental work decision must be made as to whether a given series of observed frequencies corresponds to that implied by some hypothesis, e.g., in animal or plant breeding, does the F_2 generation follow the 3:1 Mendelian ratio? Early investigators were inclined to lean heavily on 'experience' to decide whether an observed discrepancy could reasonably be regarded as fortuitous. Such a procedure usually has a high subjective bias. In 1900, Karl Pearson (75) provided an objective procedure when he published his *Chi Square* criterion for testing goodness of fit. Through further work by Pearson and

TABLE 10

Comparison of distribution of nitrogen fixed by clover plants in agar with theoretical values from normal curve

RANGE, MG NITROGEN	OBSERVED FREQUENCY f_o	THEORETICAL FREQUENCY f_t	$f_o - f_t$	$\dfrac{(f_o - f_t)^2}{f_t}$
<0.30	3	5.8	−2.8	1.35
0.30–0.45	5	6.2	−1.2	0.23
0.45–0.60	9	10.4	−1.4	0.19
0.60–0.75	22	15.6	+6.4	2.62
0.75–0.90	29	21.2	+7.8	2.87
0.90–1.05	25	24.9	+0.1	0.00
1.05–1.20	23	26.4	−3.4	0.44
1.20–1.35	20	25.1	−5.1	1.04
1.35–1.50	17	21.2	−4.2	0.83
1.50–1.65	16	16.0	0.0	0.00
1.65–1.80	11	10.7	+0.3	0.01
1.80–1.95	7	6.4	+0.6	0.06
1.95–2.10	4 } 9	3.4 } 6.1	+2.9	1.33
>2.10	5	2.7		
Total	196	196		11.02

$$n = 10 \qquad P = 0.35$$

others, notably R. A. Fisher, the field of application of the X^2 criterion has been greatly broadened and its interpretation clarified.

The Chi Square Test for Goodness of Fit. In table 10, an observed distribution of nitrogen fixed by clover plants in agar is compared with the distribution corresponding to the normal curve with mean and variance, estimated from the data, of $\bar{x} = 1.129$ and $\Sigma (x - \bar{x})^2/(N - 1) = 0.1888$ respectively. Do the observed frequencies in the respective classes, f_o, agree within the limits of sampling fluctuations with the theoretical frequencies, f_t, corresponding to this normal curve? To answer this question one calculates

[38]
$$X^2 = \Sigma \frac{(f_o - f_t)^2}{f_t}$$

when Σ denotes summing over the cells of the table. The accuracy of the test is improved by combining classes in the 'tails' to bring the theoretical frequency

up to 5 or more, as indicated in table 10. The distribution of X^2 depends on n, the number of degrees of freedom involved; tables of the significance levels of X^2 are available (30, 35, 79, 84, 98).

In this example there are, after combining, 13 classes of frequencies, so that X^2 possesses $13 - 3 = 10$ degrees of freedom, since the normal curve chosen was selected to have the same total frequency, the same mean, and the same variance thereby introducing 3 constraints which absorb 3 degrees of freedom. The value of X^2 observed, 11.02, corresponds to a probability, P, of 0.35; that is, if the distribution of nitrogen fixed *is* normal, comparison with a normal curve fitted as above would be expected to yield a X^2 as large or larger 35 times in 100. Therefore, since X^2 does not exceed the 5 per cent significance level, the test does not indicate that the normality hypothesis should be discarded. However, it should be noted that 2 of the 13 components (underlined values in table 10) contribute approximately half of the total X^2. That marked skewness of the observed distribution is responsible for this feature is apparent when the data and fitted curve are plotted (117). In such a case one is reluctant to accept the normality hypothesis even in the light of a 'favorable' value of X^2, and should reserve judgment until further data are at hand.

K. Pearson (78) emphasizes that, although the X^2 test will enable the experimenter to determine whether a given curve (or type of distribution) will reasonably describe the observations and may even allow some choice between alternative graduation curves, the 'better' curve as judged by the higher value of P does not necessarily represent the distribution from which the material was drawn. It requires a large-sized sample to discriminate between alternative curves.

A second example of the X^2 test is provided by the data in table 2 in which the distribution of bacteria on the squares of a Petroff-Hausser counting chamber is compared with that based on the proper Poisson distribution. The number of frequency classes is 8, therefore X^2 has 6 d.f. as one degree each is lost through keeping equal the population totals and the means of the two distributions. The probability corresponding to X^2 is 0.43, and we may conclude that the data do not conflict with the hypothesis that they came from a Poisson series.

A useful application of the X^2 criterion depends on the fact that a series of independent X^2's may be summed to form a total X^2 which possesses degrees of freedom equal to the sum of the degrees of freedom of its components. Thus, routine testing in a laboratory may be checked occasionally by summing the X^2 made over a period and testing the sum so obtained. Wilson and Kullman (114) used this method of statistical control to check the accuracy of counts of the root nodule bacteria made with the Petroff-Hausser chamber method under routine laboratory procedures. In each trial 144 squares were counted, and during the period the species of organism, density of suspension, and type of medium were varied. For each of the 50 trials, a X^2 was calculated by the same procedure as illustrated in table 2 and the sum tested. Since the number of degrees of freedom involved was outside the range of published significance levels, use was made of the fact that for large values of n, the distribution of $\sqrt{2X^2}$ is approximately normal with unit standard deviation about a mean of $\sqrt{2n - 1}$, so that the difference, $\sqrt{2X^2} - \sqrt{2n - 1}$ can be regarded as a normal deviate. In this particular case a normal deviate of $+0.57$ was obtained, which is well below that for the 5 per cent level of significance.

A further use of X^2 is for testing independence in a contingency table. In such a table an individual is classified in two (or more) different ways and the question is: Are the two methods of classification independent? Contingency tables provide a rapid and simple method for detecting associations in enumeration data and are of especial value when either or all of the classifications is qualitative. A 2×2 contingency table is shown in table 11. The data are from the study of McCarter, Getz and Stiehm (62) on the comparative response of different classes of students to intracutaneous injections of purified protein derivatives (P.P.D.) from the avian and human types of the tubercle bacillus. The Short Course students are boys, all from farms; the Freshmen are first-year male students at the University, predominantly from urban homes.

The 'expected' values for each cell when calculated on the hypothesis of independence require the four frequencies to be proportional; hence they can be determined from the marginal values. For example, in the first cell the expected value is $(497 \times 739)/1026 \approx 357.98$; the other values are automatically determined since the marginal totals of expected and observed must be equal. This means that the X^2 found will have only one degree of freedom. Since X^2 is 5.58 which corresponds to a P value of 0.02, the rejection of the hypothesis of independence is indicated at the 5 per cent level of significance.[33] The cause of the high value of X^2 apparently is the excess of Short Course students who reacted positively to the avian P.P.D. Further information was obtained from the responses of the students when tested with both human and avian P.P.D. The results (table 12) gave a X^2 value of 8.10; as calculation of three cells in a single

[33] The value of X^2 for a 2×2 contingency table,

a	b	$a+b$
c	d	$c+d$
$a+c$	$b+d$	$a+b+c+d=N$

may be evaluated, without calculating the expected values, from the formula

$$X^2 = \frac{N(ad-bc)^2}{(a+b)(c+d)(b+d)(a+c)}$$

When expected frequencies less than 500 occur in a 2×2 table, the 'Yates correction for continuity' should always be applied. This consists of decreasing by 1/2 the frequencies which exceed their expected values, and increasing by 1/2 the frequencies which fall short of their expected values. These steps may be shortcut by noting that

$$\text{corrected } X^2 = \frac{N(|ad-bc|N/2)^2}{(a+b)(c+d)(b+d)(a+c)}$$

where $|ab-bc|$ denotes the value of $(ad-bc)$ taken positive, so that the correction, $-N/2$, always reduces the magnitude of the quantity to be squared.

In the present case the corrected X^2 is 5.26 which is still significant at the .05 level. No satisfactory correction of this sort has been found for the general $r \times c$ table. For further discussion of this correction and the additional refinements in the case of a 2×2 table see Fisher and Yates (35).

row will fix all the other values if the marginal totals remain unchanged, there are 3 d.f.   In general, a table of r rows and c columns has $(r-1)(c-1)$ d.f. As the probability is again less than 0.05, the hypothesis of independence is doubtful.   As can be seen in the table, the largest contribution to X^2 arises from the excess of Short Course students who are human P.P.D.-negative and avian-positive.

TABLE 11

Contingency table showing response of students to avian P.P.D.*

	AVIAN +	AVIAN −	TOTALS
Freshmen	(357.98) 341 0.805	(381.02) 398 0.757	739
Short Course	(139.02) 156 2.074	(147.98) 131 1.948	287
Totals	497	529	1,026

$$X^2 = 5.58 \qquad n = 1 \qquad P = 0.02$$

* In both contingency tables the expected values for each cell is given in parentheses; the contribution of X^2 from each cell is underlined.

TABLE 12

Contingency table showing response of students to human (H) and avian (A) P.P.D.

	H+ A+	H+ A−	H− A+	H− A−	TOTALS
Freshmen	(173.63) 176 0.032	(3.43) 3 0.054	(177.05) 165 0.820	(384.89) 395 0.265	739
Short Course	(29.37) 27 0.191	(0.57) 1 0.324	(29.95) 42 4.848	(65.11) 55 1.570	125
Totals...............	203	4	207	450	864

$$X^2 = 8.10 \qquad n = 3 \qquad P = 0.045$$

D^2 *Test for Binomial Distributions.*[34]   A rapid method of testing for agreement with binomial sampling—and which is the only method practicable when

[34] The index of dispersion appropriate to the binomial distribution which we have denoted by D^2 was introduced by an author using the pseudonym "Mathetes" (66) following an analogy with previous work of Fisher, Thornton, and Mackenzie (34).   Its use as a test of sampling technique has been illustrated by Fisher (30, sec. 19); Snedecor (84, sec. 9.6); Snedecor and Irwin (85); and by others.   Cochran (19), Haldane (46) and Welch (104) have studied its sampling distribution in very small samples from binomial populations.

the samples are of different sizes—is based on the *dispersion index* appropriate to the binomial distribution, namely:

[39]
$$D^2 = \Sigma \frac{(x_i - N_i p')^2}{N_i p'(1 - p')}$$

where N_i denotes the number of individuals in the ith sample, $(i = 1, 2, \cdots, k)$, x_i is the number of the individuals in the ith sample which possess the characteristic under investigation, and $p' = \Sigma x_i / \Sigma n_i$ is the observed *proportion* of individuals with this characteristic in all the data at hand, *i.e.*, in all k samples lumped together. If all k samples are of the same size, so that $N_i = N$ for all i, then [39] simplifies to

[40]
$$D^2 = \frac{N\Sigma(x_i - \bar{x})^2}{\bar{x}(N - \bar{x})} = N\frac{\Sigma x_i^2 - (\Sigma x_i)^2/k}{\bar{x}(N - \bar{x})}$$

where $\bar{x} = \Sigma x_i/k$ is the average number of individuals with the characteristic in question per sample.

This index of dispersion, D^2, is essentially a criterion for judging whether the variance of the observed frequency distribution is in agreement with the variance of the binomial distribution *having the same mean*. When sampling is in accordance with the binomial distribution, the expected value of D^2 is $(k - 1)$, and its sampling distribution about this mean is well represented by the tabular X^2-distribution for $(k - 1)$ degrees of freedom provided k is small compared with ΣN_i, *i.e.*, provided that either the individual samples are large, or, if they are small, that there are many of them. In consequence, the tabulated significance levels of X^2 can be used for testing the statistical significance of an observed value of D^2.

For data arranged in a frequency table, such as table 1, a form of equation 39 more convenient for calculation is

[41]
$$D^2 = \frac{\Sigma f_x x^2 - \frac{(\Sigma f_x x)^2}{\Sigma f_x}}{\bar{x}(N - \bar{x})}$$

where $\bar{x} = (\Sigma f_x x)/(\Sigma f_x)$. For the data in table 1: $N = 100$, $k = \Sigma f_x = 113$, $\Sigma f_x x = 673$, and $\Sigma f_x x^2 = 4779$, yielding $D^2 = 137.612$ as compared with an expected value of 112. Since tables of the significance levels of X^2 do not go as high as 112 degrees of freedom, whether the observed D^2 is significantly greater than its expected value has to be tested here by employing $\sqrt{2X^2} - \sqrt{2n - 1}$ as a normal deviate with unit standard deviation and taking $X^2 = D^2$ and $n = k - 1$. In the present instance, $\sqrt{275.224} - \sqrt{223} = +1.66$ which is

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These writers, with the exception of Welch, denote this index of dispersion by X^2; Welch uses D. We have used D^2 to avoid confusion with the X^2 goodness-of-fit criterion, adopting D^2 instead of D because the former carries the implication that its value is always positive or zero.

just significant at the .05 level.[35]   In brief, the variance of the observed frequency distribution exceeds significantly the variance of the binomial distribution with the same mean.

In the preceding example, the observed D^2 was significantly larger than the value expected on the hypothesis of binomial sampling.   Although values of D^2 may frequently be obtained which are less than the values expected in binomial sampling, generally these will not differ widely from the expected value.   If, however, values considerably less than the expected values occur, the significance of the discrepancies may be judged by noting whether D^2 is *less* than the 0.95 level of χ^2.   Should such 'significantly small' values of D^2 be a common occurrence, it is well to seek an explanation of the unusual uniformity of the samples. For example, had a significantly small value of D^2 been obtained in the study on monocytes (table 1), one might have inquired whether some physiological factor controlled their distribution in the blood so that this distribution was more uniform than would occur in random mixing.

SUMMARY

On the basis of the discussion given in the main body of the text the following points should be emphasized:

1. Statistical analyses provide no substitute for proper and precise experimental technique.   The quantitative relationships to be derived are not altered through any statistical magic; their accuracy is that of the observations.   As Fisher has said, "The statistician must be treated less like a conjurer whose business is to exceed expectation, than as a chemist who undertakes to assay how much of value the material submitted to him contains" (Rothamsted report for 1933).   As was illustrated in several instances, however, statistical analysis can be of great assistance in providing a measure of the adequacy of a particular method used in practice.

2. Although statistical analysis will divulge only those facts present in the observations, such analysis furnishes a tool for extracting information inherent in the data but not readily evident by mere inspection.   As a corollary to this, statistical methods allow quite complicated experiments to be designed in which the influence of each of several variables on some particular phenomenon as well as their interactions can be simultaneously determined.   Such complex experiments are extremely valuable for saving of time, labor and money, and

[35] We are testing here whether D^2 significantly *exceeds* its expected value so the .05 significance of D^2 is judged by Fisher's .10 significance level (1.64485) for a normal deviate with unit variance.   The reader with some statistical experience may be curious to know why, that we have gone to the trouble of finding the expected frequencies, we do not test the agreement of the observed and expected frequencies with the χ^2-test of goodness-of-fit. To meet the conditions of applicability of the χ^2-test we should have to group together (as indicated in table 1) several of the frequencies at the tails on account of the small numbers involved, thereby reducing the sensitivity of the χ^2-test.   Fisher (30, sec. 19) discusses the relative usefulness of the two tests.

provide a truer representation of the actual situation than several experiments in which the several factors are varied one at a time.

3. Statistical considerations will suggest the proper design of an experiment— proper not only in the sense that the results are most readily amenable to statistical treatment, but also from the viewpoint of economy and efficiency. A corollary to this advantage is that the statistician should be consulted before the experiment is performed rather than asked to do the impossible: make valid conclusions from the data of a poorly designed experiment.

4. Statistical theory emphasizes the necessity of repeating an experiment. Statistical analysis is of little use on the individual experiment unless something is known about the properties of the population; usually this is most accurately obtained through repeated trial under similar circumstances. Although most experimenters appreciate the fact that results "have weight" only when they have been obtained in several experiments, without recourse to statistical considerations it is often difficult to determine how many repetitions are needed. Often, by statistical procedures, the experimenter may decide beforehand by using his previous experience whether the results of a contemplated investigation will be worth the effort, time and expense required to obtain an unequivocal answer.

5. Statistical measures in conjunction with statistical theory provide a means for condensing information derived from large-scale experimentation.[36] The essential information in a large mass of data, whose tabulation would require much space and whose very size may intimidate a reader wishing to make his own interpretation, can often be summarized in the form of a few statistical measures (e.g., number of observations taken, mean with its standard deviation, a regression coefficient with its standard error, an analysis of variance) with little loss of relevant knowledge.

APPENDIX AND EXPLANATORY COMMENTS

As with other branches of science, statistics possesses a technical vocabulary in which common words are used in a sense which often proves confusing to those unfamiliar with the special meaning. Many of these are concisely defined by mathematical formulas which are supplied in the text; others need descriptive explanation which is given in the following glossary of terms:

Argument: one of the independent variables upon which a tabled function depends, the values of which are given at the margin of the table and make it possible to locate the corresponding tabled values, e.g., in a table of logarithms, the function tabled is $\log N$ and N is the argument.

Confidence Intervals and Fiducial Limits: it has long been realized that a single value computed from a sample as an estimate of a parameter θ has very little chance of actually equalling θ, and that some sort of range to indicate the probable accuracy of the estimate is needed. Thus, with an observed proportion, p_o, the estimate of the true proportion, p, was given as $p_o \pm \lambda\sigma_o$ where $\sigma_o = \sqrt{(p_oq_o)/N}$ and λ was chosen to correspond to some probability P, and a statement often made was: "The probability the true proportion, p, lies outside the limits $p_o - \lambda\sigma_o$ and $p_o + \lambda\sigma_o$ is less than or equal to P." So far as we have been

[36] Further discussion and illustration of this point are given in the *A.S.T.M. Manual on Presentation of Data*, American Society for Testing Materials (260 S. Broad St., Philadelphia, Pa.), 3rd printing, August, 1940.

able to determine, E. B. Wilson (112) first pointed out that such a statement is erroneous. He stressed that it is p_o which is a chance variable, not p, and that a correct statement must take some such form as: If it is not true that $p_l < p < p_u$, where p_l and p_u depend on p_o and P, then the probability of our observing the value p_o itself, or any more improbable value, is less than P.

Wilson showed in the above case how to calculate the limits p_l and p_u , but did not follow up his note with a general discussion of the procedure to be followed in other cases. The first general treatment was that of R. A. Fisher(32), who appears to have been unaware of Wilson's note. Fisher terms such limits *fiducial limits*, and his development is in terms of what he calls *fiducial probability*. About 1930, J. Neyman independently initiated his theory of *confidence intervals* (see ref. 70, where a list of the principal papers on fiducial limits and confidence intervals is given) and for some time it seemed to many statisticians that the two approaches were equivalent, since in the cases considered they had led to identical ranges for the parameter being estimated. A further paper by Fisher (33b) threw doubt on the equivalence of the two approaches, which doubt increased as more papers appeared. The two approaches have been contrasted by Neyman (70), and, while they lead to identical results in many instances, there are cases of disagreement, so they should no longer be regarded as equivalent. Neyman's approach seems to be more general, and for this reason, as well as from a greater familiarity with it, we have adopted the method of confidence intervals in the present paper.

Degrees of Freedom: the number of variates upon which a quantity depends minus the number of constraints upon these variates, *e.g.*, if N independent x values are concerned, then $\Sigma(x - c)^2$ has N degrees of freedom provided c is in no way determined by the values of the x's themselves; on the other hand, $\Sigma(x - \bar{x})^2$, where $\bar{x} = \frac{1}{N}\Sigma x =$ average of x values, has only $(N - 1)$ degrees of freedom since the quantities $(x - \bar{x})$ are constrained by the equation $\Sigma(x - \bar{x}) = 0$.

e: base of natural logarithms; is a constant like π. Common logarithms use 10 as a base. In this paper ln signifies that the base is e, log, that the base is 10.

Entry: a quantity appearing in the body of a table, *e.g.*, the tabled values of log N are entries.

Likelihood (as introduced by R. A. Fisher): the likelihood of a particular value θ' of a parameter θ in the light of an observed sample, is proportional to the probability of this sample when $\theta = \theta'$; the largest likelihood can be assigned the value unity by convention, if desired; in comparing two particular values, θ' and θ'', of a parameter θ, importance attaches only to the ratio of their likelihoods, not to the values of the respective likelihoods. Thus, while we may know nothing about the relative frequency with which $\theta = \theta'$ and $\theta = \theta''$ in a particular type of research, it may be an inescapable fact that the probability of the observed sample is three times as great when $\theta = \theta'$ as when $\theta = \theta''$. "If we need a word to characterize this relative property of different values of [θ], I suggest that we may speak without confusion of the *likelihood* of one value of [θ] being thrice the likelihood of another, bearing always in mind that likelihood is not here used loosely as a synonym of probability, but simply to express the relative frequencies with which such values of the hypothetical quantity [θ] would in fact yield the observed sample." (28, p. 326). Likelihood also differs from probability in that it is not capable of being summed or integrated; whereas the sum of the probabilities of all possible values of a random variable is unity, the sum of the likelihoods, computed from a particular sample, of the admissible values of a parameter will in general be infinite. An exact knowledge of the likelihood of different values of θ tells us nothing whatever about the 'probability' that θ will fall in any given range. Indeed, in well conducted research, θ will not have a probability 'distribution', but will be confined to a single value, the identity of which is unknown, and the object of the research will be to estimate this value as closely as possible.

Method of Maximum Likelihood: estimating a parameter from a sample by choosing the value of the parameter which has the largest likelihood, as defined above, when calculated

from the sample at hand; the estimate so obtained is termed the *optimum estimate* of the parameter, or its *maximum likelihood estimate*.

When the parameter θ can itself be regarded as a random variable drawn from a super-population in which all values of θ are equally probable *a priori*, then the value of θ which has the highest 'probability' of 'producing' the observed sample—this *a posteriori* 'probability' being calculated with the aid of Bayes' Theorem—is identical with the maximum likelihood estimate of θ from this sample. This property was employed by Gauss in 1809 to justify his development of the method of least squares by a formulation identical with that now used in the method of maximum likelihood. Later (1829), however, he gave less stress to this argument through the conviction that maximizing the probability was less important than minimizing the injurious effects of the actual errors of estimation (see Fisher, (33b) p. 249).

The justification of the method of maximum likelihood by Fisher and his followers comes from this later viewpoint. Fisher (28, 30a) has shown that, of the estimates calculated from large samples, the one obtained by maximizing the likelihood is in general the one for which the intrinsic accuracy is greatest, and that, when there exist estimates, called *sufficient statistics*, which exhaust the information in the sample about the parameter, then these are the maximum likelihood estimates. Thus, use of the method of maximum likelihood is justified by the advantageous properties of the estimates to which it leads. Further elaboration can be found in two expository papers by Fisher (33a, b), the former being supplemented by the critical comments of many noted statisticians, of which J. Neyman's are especially interesting.

Null Hypothesis: a hypothesis, relating to the parent population(s) of the research data under consideration, and whose acceptance or rejection is to be based on the agreement or non-agreement between some of its logical consequences and corresponding aspects of the research data. Obviously a null hypothesis must be sufficiently specific to permit the deduction of some criterion of agreement between observations and this hypothesis. For example, the assertion that a drug "reduced the mortality" from a certain disease "*10 per cent*" could constitute a null hypothesis. Mere assertion that it "reduced the mortality" could *not* constitute a null hypothesis, however, since this hypothesis is not sufficiently specific to permit an evaluation of the agreement of observations with the hypothesis. In this case, the null hypothesis is usually taken to be that the drug produced *no reduction* in mortality from the disease, the contradiction of which by experimental results would lead to the inference that *the treatment reduced the mortality*.

Parameter: in the strictest sense, a constant in the mathematical formula specifying a hypothetical population the value of which serves to distinguish a specific population from others having the same functional form, and by varying the value of which different populations of the same general family can be specified, *e.g.*, m, is the parameter of the Poisson distribution, the general term of which is

$$\frac{e^{-m}m^x}{x!}$$

More generally, any descriptive quantity such as an average, correlation coefficient, *etc.*, relating to a population and which serves to distinguish this population from other conceivable populations.

Parent Population: used in connection with a particular sample to designate the population from which it was drawn.

Population or Universe: the aggregate of all individuals or objects which, by reason of some characteristics in common, may logically be regarded as comprising the set of objects under consideration. When the objects are susceptible of complete enumeration, the population is *finite*; otherwise, *infinite*. Thus, a set of laboratory animals constitutes a finite population, whereas the temperatures at which a species of bacteria will grow forms an infinite population. A finite population may consist of so large a number of items as

to permit being treated as infinite without sensible error, *e.g.*, yeast cells growing in a vat. The foregoing refers to *real* populations. Often *hypothetical* populations are employed. An example is the abstract population of offspring which a geneticist regards as producible by repeated matings of specified character, although some individuals of such a population can never be produced; furthermore, the population exists in the abstract even if no matings are carried out at all.

In bacteriology it is often desirable to regard a quantity of liquid as a population, *e.g.*, a sample of water or milk. In such cases it appears somewhat artificial to regard the molecules as the individual objects comprising the population, and it seems more natural to regard it as an aggregate of arbitrary volumetric units of the liquid, such as milliliters. Although the latter approach is often convenient, two aspects of it should be noted: the arbitrary size of the constituent 'objects' and their momentary identity under any sort of mixing process.

Random: an operation, such as drawing a sample from a population or arranging pots in a greenhouse, is performed in a *random* manner when its execution is such that *a priori* each and every possible outcome has an equal chance of occurrence. Thus, a sample drawn from a population in such a manner that *a priori* each and every individual of the population has an equal chance of being included is termed a *random sample*. Since any sample whatever can be obtained either by a random or by a non-random operation, it is important to note that *it is the operation of drawing the sample which is random and not the sample itself.*

The presence of randomness, in sampling and in laying out experimental arrangements, is required for the validity of statistical tests of significance. Accordingly, operations which are to be performed at random should be faithfully randomized with the aid of a table of random numbers, dice, drawing cards out of a hat, *etc.*—mere absence of conscious system is not enough to insure freedom from bias. For illustrations of sampling bias arising in samples selected without attention to randomization, see Cochran and Watson (22) and Yates (122).

Replicates: the subdivisions of an experiment which are similar with respect to some factor under investigation although they may be dissimilar with respect to other pertinent characteristics. For example, the animals inoculated with a particular one of several organisms being tested constitute the replicates of that inoculum although they may come from different litters or correspond to different age groups, *etc.*

Σ: the sum of, *e.g.*, if there are N values of x, then $\frac{1}{N} \Sigma x$ denotes the average (arithmetic mean).

Sample: a finite portion of a population.

Statistic: any quantity calculated from an observed sample with a view to characterizing the parent population; a value calculated from a sample as an estimate of a parameter of the parent population, *e.g.*, the mean of a sample is a statistic in that it provides an estimate of the mean of the parent population. A statistic used to estimate a parameter need not be the quantity in the sample which directly corresponds to the parameter in the population, *e.g.*, the range of a sample (*i.e.*, largest observation minus the smallest) provides an estimate of, and hence is a statistic for, the standard deviation of the population, as is also the standard deviation of the sample. Infinitely many statistics could be devised for estimating a particular parameter from an observed sample, but *comparatively few of these* would be of practical utility, and of these there are often strong theoretical reasons for a particular choice.

Statistically Significant: a discrepancy between some property of a sample and that which is expected on the basis of a particular null hypothesis is *statistically significant* if the probability of a discrepancy as bad or worse arising solely from sampling fluctuations (*i.e.*, from errors of measurement, biological variation, *etc.*) admissible under the null hypothesis is less than some preassigned quantity, α, known as the *level of significance.* The value of α usually adopted is 0.05, and discrepancies statistically significant on this basis are said to be *significant at the .05 (or 5 percent) level of significance.* Other values of α

are also used; the desiderata to be taken into consideration in choosing a level of signifi-cance for a particular line of research are discussed in the text. Early writers used P to denote the level of significance, and this notation is still employed by many biologists, but the use of α for this purpose has definite advantages when tests of significance are con-sidered from the viewpoint of Neyman and Pearson, so that today α is employed by the majority of American writers on mathematical statistics.

It should be noted that the statistical significance of a result depends solely on the probability of occurrence of results equally or more discrepant as a consequence of sampling fluctuations admissible under the null hypothesis, and is not in itself an indication of the practical signif-icance of the result. For example, an observed correlation coefficient, r, of .10 calculated from a sample of 500 is significantly different from zero at the .05 level and suggests the existence of a real correlation of the order of .10 between the variables under consideration. If the *complete independence* of these variables is important, this contradictory evidence is of practical significance. On the other hand, if it is desired to predict the values of one variable from that of the other, this correlation is practically worthless, for it means that $100(1 - r^2) = 99\%$ of the variation in each variable is independent of variation in the other.

Variable: a quantity which in a given context can assume different values in different individual cases; the antithesis of a constant, the latter being a quantity which in a given context can have only a single value (which may or may not be known.)

Variate: a variable; when used in the plural the word often refers to the particular values of a single variable corresponding to individual cases, especially when these values are unknown, *e.g.,* the heights of ten individuals, denoted by x_1, x_2, $\cdots$, x_{10}, are ten variates, but height symbolized by x is the only variable.

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FACTORS INFLUENCING THE ENZYMIC ACTIVITIES OF BACTERIA

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CONTENTS

The study of the metabolism of bacteria differs from that of the animal tissue cell in that the physical and chemical conditions holding in the environment in which the bacterial cell is formed have to be taken into account. The animal tissue cell is formed and metabolizes in an environment which is stabilized within narrow limits compared with the wide range of temperature, pH, oxygen tension and chemical conditions to which bacteria are subjected and under which they can exist and multiply satisfactorily. The animal body is a highly evolved system which has acquired complex regulating mechanisms ensuring that its constituent cells shall not be subject to any gross changes: thus we have the system of buffers in the blood working in conjunction with respiratory and kidney mechanisms for the stabilization of blood pH, the delicate nervous regulation of body temperature, and the regulation of the chemical constitution of the blood by kidney action. The bacterial cell belongs to a form of existence which has evolved few, if any, mechanisms for the control of its external environment but which reacts to a change in physico-chemical surroundings by an alteration in its enzymic constitution so that the resultant change in the internal environment shall be as small as possible and so that the cell can continue to metabolize under the changed conditions. The idea that the bacterial cell has a fixed enzymic constitution has been discarded for some time; and the object of this review is to outline the types of variation that the enzymic constitution may undergo in response to alterations in the external environment and to deduce, so far as is possible at present, the principles underlying these variations.

There is a limit to the total range of enzymes that any one organism can produce, and one organism may react to a given change in conditions in a way specifically different from another. Systematic bacteriology makes use of such differically different from another. Systematic bacteriology makes use of such differ-

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ences for the separation of genera, species, and strains. Thus every organism has a repertoire of activities which it can produce but the particular activities selected from the repertoire for inclusion in any one culture of that organism are determined by the conditions holding during the growth of that culture. It is intended to draw the distinction here between the potential enzymic constitution, representing the repertoire of an organism, and the actual enzymic constitution which is that section of the potential constitution which is produced in response to a given set of growth conditions.

Our knowledge of the interplay of growth conditions and actual enzymic constitution is steadily growing larger. Much of the knowledge has been acquired through work with washed suspensions of organisms and as such must be regarded as limited by the restrictions of that technique. Any study of enzyme constitution carried out with intact cells is necessarily limited by the activities of other enzymes within the cell, permeability of the cell membrane towards the substrate, differences between the internal and external environment, *etc.*, and it can rarely be stated with certainty that an observed alteration in activity is due to an alteration in enzyme-content of the cell alone. On the other hand, studies using non-viable cell preparations or cell-free fractions are limited by uncertainty regarding the amount of damage involved in the preparative treatment. While we are mainly concerned with the response of bacteria towards their environment, it is probable that results obtained with washed suspensions give a reasonably true picture of the alteration of such activities with altered such conditions, but it must be realized that such information relates to the enzymic activity displayed in the intact cell and not necessarily to the cell-free enzyme. Accordingly it is intended to use the term "enzyme activity" to cover all cases of variation studied which have not definitely been proved to be due to an alteration in the amount or properties of the actual enzyme concerned and which may be partly attributable to other factors such as permeability, *etc.*

The subject is most conveniently treated under three main headings:

Variations due to chemical factors in the growth medium.

Variations due to physical factors involved during growth.

Factors apparently related to the organism itself.

Generally speaking these factors are quite independent and the variations of enzyme activity imposed by one set of factors may be modified by the superposition of another set of variables. Thus the experimental investigation of the effect of any one factor is not fully significant unless all other factors known to affect the enzyme in question are in their optimal condition for that enzyme It is to assist in the planning of such work that this review has been constructed.

VARIATIONS DUE TO CHEMICAL FACTORS IN THE GROWTH MEDIUM

1. Presence of the specific substrate

This aspect of the subject has been reviewed in detail (57, 109, 18) and so will be dealt with here in outline only. Karström (56) studied the relation between the fermentative properties of *Betacoccus arabinosaceus* and the nature of the sugar present during the growth of the organism. He found that some sugars,

such as glucose and sucrose, are fermented whatever sugar is present during growth or if the organism has been grown in the complete absence of carbohydrate. On the other hand, the power to ferment sugars such as galactose, arabinose, lactose, *etc.* is acquired only if the specific sugar involved is present during growth. From these findings Karström divided bacterial enzymes into two groups which he defined as follows:

> *Adaptive enzymes* which are produced only when required or whose formation is dependent upon adaptation of the organism to a specific substrate.

> *Constitutive enzymes* which are formed by the cell independently of the composition of the medium in which it is grown.

The adaptive nature of certain enzymes concerned in the fermentation of carbohydrates was shown to be due to an actual appearance of enzyme in the cell in response to the presence of the substrate during growth by confirmation of the nature of the results after the cells concerned had been dried, treated with toluene, *etc.*

Many adaptive enzymes have been investigated in detail since the pioneer work of Karström; among these may be mentioned galactozymase of yeast (92) and of *Escherichia coli* (88); formic hydrogenlyase of *E. coli* (90, 91); "tryptophanase" of *E. coli* (27, 44); cysteinase of *E. coli* (17); hyaluronidase of *Clostridium welchii* (74); creatinase of certain soil bacteria (20), and the enzyme produced by an organism isolated by Dubos and Avery (19) which adaptively hydrolyzes the specific polysaccharide of Pneumococcus Type III.

Since the introduction of more quantitative methods of enzyme estimation, it seems that the classification as set out above is an over-simplification. As methods of estimation become more delicate it appears that many of the "strictly adaptive" enzymes are actually formed to a small extent when the organism is grown in the complete absence of the specific substrate. Thus, although *E. coli* which has been grown in the presence of galactose, will ferment this sugar at a rate represented by a $Q_{\text{galactose}}$ (= μg galactose removed per hour per mg dry weight organism) of 500, the organism grown in the complete absence of galactose will ferment this sugar with a Q value of 20 (88). Again, *E. coli* grown in the presence of tryptophan is able to oxidize this amino-acid to indole through the "tryptophanase" system but, according to Fildes (27), the non-adapted organism still possesses some "tryptophanase" activity, although this finding has been questioned by other workers (23). *Streptococcus faecalis* can decarboxylate tyrosine to tyramine if grown at an acid pH in the presence of tyrosine, but the organism grown in the absence of tyrosine still has an activity equivalent to approx. $\frac{1}{30}$ the activity of the adapted organism (32). Thus adaptation appears to be the result of the stimulation by the specific substrate of the production of a constitutive enzyme normally present in very small degree. To explain this adaptation Yudkin (109) has proposed a theory of enzyme production by the mass-action effect of the substrate acting upon an enzyme precursor. It is thus reasonable to speak of the constitutive and adaptive parts of an enzyme. A point that has not received much attention is whether the presence of the substrate has an "all or none" effect on the production of the adaptive part of such

an enzyme or whether the degree of adapted activity is related to the substrate concentration during growth.

The constitutive enzymes are those which, by definition, are formed independently of the composition of the growth medium. It is doubtful whether any enzyme is completely unaffected by changes in the composition of the growth medium, but the sense of the definition is obviously: "an enzyme which is formed during growth in the absence or presence of the specific substrate." A strictly constitutive enzyme would be formed to the same extent whether the substrate is present or absent during growth, other conditions remaining constant, and this is occasionally the case, e.g., the enzymes involved in the oxidation of cadaverine, putrescine and agmatine by *Pseudomonas pyocyanea* (34). Karström (57) gives glucozymase of *E. coli* as a typically constitutive enzyme, but the glucozymase activity of this organism is approximately doubled by the addition of glucose to the medium during growth (88) so that such constitutive enzymes may undergo adaptive variation. Further the constitutive enzymes may undergo wide variations as a result of the presence during growth of substances other than the specific substrate and as a result of changing physical conditions. Quastel (78) suggests that constitutive and adaptive enzymes do not represent two classes but rather the limits of variation of enzymes in the cell,—the constitutive having the least range and the adaptive, the greatest range of variability. He suggests further that the formation and destruction of "enzymes" is dependent upon the same physico-chemical laws as those controlling the metabolism of any other organic metabolite of the cell. It is obvious, however, that there is some considerable difference between the formation of formic dehydrogenase and that of formic hydrogenlyase in *E. coli*, and it is desirable that we should have some way of expressing this difference. The terms "constitutive" and "adaptive enzymes" have a definite place in the nomenclature of bacterial metabolism but do require more strict definition than before. It is necessary to know, when investigating any bacterial activity, whether that activity is going to be significant if growth occurs in the absence of the specific substrate, or whether the activity is adaptive for practical purposes though constitutive academically. Accordingly it is suggested that the terms adaptive and constitutive (a) be restricted to the relation between the production of the enzyme and the presence or absence of the specific substrate during growth and (b) be used to distinguish between enzymes which undergo gross or little variation respectively as a result of the presence or absence of the substrate during growth. Thus, adaptive enzymes are those enzymes whose production is markedly increased (Karström suggests five-fold increase as the limiting value) by the presence of the specific substrate during growth, other conditions being optimal for the production of those enzymes. Similarly constitutive enzymes are those enzymes whose production is not significantly increased (i.e., less than doubled) by the presence of the specific substrate during growth, other conditions being optimal. These definitions cannot be applied to enzymes such as glucozymase in *E. coli* which is functionally active when the organism is grown in the absence of glucose but whose activity is

doubled when glucose is added to the growth medium.   These enzymes may conveniently be referred to as "semi-adaptive".

The type of adaptation so far discussed occurs within the time required for one cultivation in the new chemical environment, but Massini (73) has shown that another type of adaptation to substrate can occur in some organisms and that this new type takes several subcultivations under the changed condition to become established.   *Escherichia coli mutabile* exists in two forms, one of which is able to ferment lactose and the other which appears to have lost this property.   If the strain which cannot ferment lactose ("white" strain) is passed through several subcultivations in the presence of lactose, then it slowly acquires the power to ferment this sugar.   Yudkin (109) differentiates between this slowly acquired adaptation and the immediate response described above by suggesting that the former process is one of "training" the organism to the new condition.   Lewis (69) showed that cultures of *E. coli mutabile* actually consist of mixtures of lactose-fermenting and non-fermenting variants whatever the growth medium, that cultures of the "white" strain grown normally contain about 1 lactose-fermenting variant in 10^5 cells, and that growth in the presence of lactose favors the production of the lactose-fermenting variants so that, by a process of selection, a culture grown through several *passages* in lactose becomes predominantly lactose-fermenting, and *vice versa*.   The training process is thus explained by the presence of the specific substrate selectively promoting the growth of the variant equipped with the enzyme necessary to deal with that substrate.   The question then arises whether the variants differ in the possession or non-possession of the enzyme lactase, or in the degree of accessibility of the substrate to the enzyme.   This question has received considerable attention by Deere (15, 16) who has shown that the white strains of *E. coli mutabile* are able to ferment lactose to the same extent as red strains if they are first dried, treated with acetone or various antiseptics, *etc.*   The evidence suggests that non-fermenting cells become fermenting if they are treated in such a way as to denature the cell protein or disintegrate the cell membrane.   It appears from this work that the white strains actually contain as much lactase as the fermenting red strains but that the lactose is unable to penetrate the cell membrane in the former case, while subcultivation in the presence of lactose leads to an increased permeability so that fermentation eventually occurs.   To reconcile this work with that of Lewis, it is necessary to postulate that the variants present in all cultures, as shown by Lewis, differ only in their degree of permeability towards lactose and that the "training" consists of promotion of the growth of the more permeable variety by the presence of lactose.

Other apparently similar cases of training occurring in the fermentation of carbohydrates have been described (7).   Fildes, Gladstone and Knight (28) have shown that certain strains of *Eberthella typhosa* are unable to grow in the absence of tryptophan preformed in the medium, but if they are serially subcultivated into synthetic media containing progressively less tryptophan, then they acquire the power to synthesize this amino-acid and become non-exacting
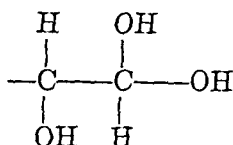
in their growth requirements. Similarly, Silverman and Werkman (83) have shown that strains of *Propionibacterium pentosaceum* are unable to synthesize vitamin B_1 which they require as a growth factor, but they can be trained in a similar manner to synthesize the vitamin and consequently to dispense with it in the growth medium. There is no evidence concerning the part played by permeability variations in these synthetic disabilities; but Kocholaty and Weil (65) have shown, in an examination of certain extracellular enzymes, that some cases of training cannot be explained on a permeability basis. They have shown that *Clostridium histolyticum* can be trained through several sub-cultivations in the presence of gelatin or casein to produce a gelatinase or casein-ase respectively; the gelatin-trained organism cannot grow in casein while the casein-trained organism is unable to grow in gelatin unless the amino-acids which are present in casein but not in gelatin are added to the medium. They "picture the differentiation between constitutive and adaptive (trained?) enzymes ·s a function of time: the longer a micro-organism is trained under altered environmental or nutritional conditions, the more will the properties of the constitutive enzymes disappear until finally complete adaptation occurs." It may well be that the difference between true adaptation and training adaptation is a function of time but since their experiments were not dealing with strictly constitutive enzymes, it is difficult to reconcile the rest of their statement with the facts.

Whereas true adaptation occurring within a single cultivation is an example of the selective action of the growth conditions on the potential enzyme constitution of the organims, it is more difficult to interpret the changes occurring as a result of training. The work of Deere, quoted above, on the variants occurring in *E. coli mutabile* shows that in this case at any rate the effect of training is on the permeability of the cell membrane rather than on the enzyme constitution of the individual cell, but trained alterations in extracellular enzymes would seem to involve variations in the potential enzymic constitution of the cells. Further work is obviously required on this point and until we learn what it is that decides and limits the potential enzymic constitution, speculation on this point is useless.

2. Presence of substances other than the specific substrate

This aspect of the subject has not been studied in much detail and may well play a larger part than has been suspected in the past. It is a common finding that the production of an adaptive enzyme is greater on a complex fully nutrient medium than on a simple synthetic medium containing only growth essentials and specific substrate. To take an example from the author's experience, *S. faecalis* produces practically no tyrosine decarboxylase when grown on a synthetic salt-glucose-marmite medium; the addition of tyrosine to the medium raises the activity from $Q_{CO_2} = 8$ to 20, in a specific case, but the same organism growing in a casein digest containing tyrosine has an activity of over 200. In this case substances other than the specific substrate play a part in the production of the enzyme. One idea, originally put forward by Jacoby (55), is that certain

substances are necessary to provide the unsynthesizable parts or "Bausteine" of the enzyme structure. Jacoby studied the production of urease by *Proteus vulgaris* and came to the conclusion that the group

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-\text{C}\!\!-\!\!-\text{C}\!\!-\!\!-\text{OH} \\
\quad\ | \qquad | \\
\quad\ \text{OH}\quad \text{H}
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$$

provided by certain carbohydrates in the medium is essential for the formation of the enzyme molecule. Jacoby's results were not quantitative and reinvestigation of the problem by Passmore and Yudkin (75) failed to confirm his results. In any investigation of the effect of carbohydrate in the medium, the change in pH produced by fermentation must also be taken into account in the interpretation of the results, and it is seldom possible to state categorically that an effect is due to the presence of any certain carbohydrate as such. However, we have an idea similar to that of the "Baustein" hypothesis involved in the modern theory of growth factors which appear to act in certain cases as coenzymes and hence exert an important effect on enzyme activity. The action of growth factors will be discussed separately below.

The presence in the medium of optimal quantities of certain metallic ions may have a marked influence on enzymic activities of the organims. Thus we have the action of Ca ions in stimulating the production of gelatinase by *P. vulgaris* (40 to 42); of Mg on the phosphatases of the propionic acid bacteria (76), and of the various ions found by Burk (5, 6) to play an important part in the fixation in N by *Azotobacter*. Recently Davies (12) has found that *Clostridium acetobutylicum* is unable to ferment maize-meal if this is first treated in such a way as to remove potassium. The full fermentation can be restored by the addition of M/1000 K to the growth medium; it is possible that the effect is one on growth rather than on fermentation, although the recent work (68, 77) indicating that K ions are intimately involved in the utilization of carbohydrate by microorganisms, would suggest the latter. The actual function of the ions is not understood in the majority of these cases although Mg has long been regarded as a coenzyme of muscle phosphatase.

Most of the investigations that come under this heading have dealt with the effect of glucose or other fermentable carbohydrate in the medium on the production of various enzymes. The investigations have centred mainly on the enzymes concerned in the breakdown of proteins and amino-acids by bacteria. Kendall and his co-workers (60 to 62) studied the production of ammonia by many bacterial species when growing in protein digests and showed that the addition of carbohydrate to the medium resulted in a decrease, even amounting in some cases to complete inhibition, of ammonia formation. They attributed the effect to a protein-sparing action of the glucose on lines similar to those then postulated in mammalian metabolism, and this argument has doubtless been the cause of the restriction of such investigations to the breakdown of proteins and amino-acids. Epps and Gale (25) have shown, however, that the inhibitory

effect of glucose in the medium is not restricted to such enzymes but applies also to some dehydrogenases, *etc.* They also found that cultures of *Micrococcus lysodeikticus* grown in a glucose-casein-digest broth are apparently unable to metabolize glucose at a significant rate and that the presence of glucose in such a medium has no action on the catalase, urease and fumarase contents of the cells. On the other hand Quastel (78) has reported that when this organism is grown on a solid peptone-agar medium, the inclusion of glucose results in the inhibition of catalase and fumarase formation and the stimulation of urease formation.

One point at issue is whether glucose produces these effects directly by action on the enzyme or its production, or whether they are due to the action of some less specific changes brought about by the presence of glucose. In most cases where glucose is metabolized, its presence in the growth medium has two marked results: first an increased crop of organisms, and second, the production of acid and consequent alteration of the environmental pH. Recently, it has also been demonstrated that the presence of glucose in the growth medium leads to a transient deposition of polysaccharide within the cell (14, 68, 77). Before it can be postulated that glucose itself causes the changes in enzyme activity, the possibility that they are due to these, or other, coincidental changes must be disproved.

Berman and Rettger (4) investigated the effect of the presence of glucose on the production of proteolytic enzymes by bacteria. They found that the production of these enzymes is inhibited in those organisms which carry out a vigorous fermentation with the formation of acid, but that glucose has little effect on the proteolytic activities of organisms with little or no fermentative powers towards this sugar. Further, if steps are taken to neutralize the acidity due to fermentation by strongly buffering the medium, then the inhibitory effect is checked. This work would seem to indicate that the inhibitory effect of glucose on protease production is due to the effect of the acidity evolved during fermentation.

Raistrick and Clark (82) pointed out that ammonia is not only a product of the decomposition of porteins but also a source of nitrogen for growth. Since the addition of carbohydrate to the medium results in an increased production of cell-N, it is only reasonable that the amount of ammonia-N liberated should be decreased by such addition. This is undoubtedly true and has been the subject of other detailed investigations (99), but in the majority of cases the difference in the ammonia formation in the presence and absence of glucose is much larger than can be explained by the greater production of cell-N in the former case. Stephenson and Gale (89) investigated the effect in respect to the deaminases of glycine, alanine and glutamic acid in *E. coli.* Using washed suspensions prepared from cultures grown in casein digest with and without glucose, they showed that (a) the addition of glucose to the washed suspension has little or no effect on the deamination processes carried out by that suspension, and (b) suspensions prepared from bacteria grown in the presence of glucose have about 5 per cent of the deaminase activities of suspensions grown in the absence of glucose. This makes it clear that glucose has no effect upon the metabolism of the organism

once the cell has formed its enzymes but that the action must be upon the formation of the enzymes during growth. These observations were later extended to include the deaminases of *dl*-serine (37), and aspartic acid (29). The inhibitory effect was not altered by bubbling the cultures with oxygen and so is not due to anaerobiosis produced by fermentation gases. An attempt was made to control the pH of the medium during growth by the addition, with constant stirring, of chalk but this measure does not necessarily have appreciable control of the pH within the fermenting cell. In any case it was without effect on the inhibitory action. One way of distinguishing between the action of glucose as such and of the acidity it produces is a comparison of the activities of organisms grown in the presence of glucose with those of organisms grown in a non-carbohydrate medium adjusted to the pH value which is finally attained in the glucose medium. This has been done by Epps and Gale (25) working with *E. coli* which attains a final pH of 5.2 when grown in 2 per cent glucose-casein-digest broth at 27 C. Some of the results are set out in table 1. It can be seen that the addition of glucose to the growth medium makes some variation in the activity of every enzyme studied if this activity is compared with that of organisms grown in non-carbohydrate media adjusted to pH 7. Similarly, alteration of the pH for growth from 7 to 5 also affects the activities. The nature and significance of these variations is discussed below. The enzymes can be classified in three groups according to the effect produced upon their activities by the presence of glucose during the growth of the organisms containing them.

In the first group of enzymes in table 1, the alteration in activity produced by the addition of glucose is the same as that produced by an acid culture medium and there is no need in these cases to postulate any action due to glucose other than that due to the acidity resulting from its fermentation. In the second group of enzymes, the presence of glucose has an inhibitory action which is greater in every case than can be explained by the acidity it produces. The effect is very marked in some cases, such as aspartase, alcohol and formic dehydrogenases, where cultures grown at pH 5 have higher activities than those grown at pH 7 while those grown in glucose have activities much less than those of the pH 7 cultures. Here, then, the action of glucose cannot be attributed to acidity. The position of the deaminases, other than aspartase, in table 1 is not so clean cut as it is with certain other enzymes, but it has been found that the low activities of the glucose cultures are not raised to the smallest degree if the culture is maintained at pH 7 throughout the growth and fermentation by the controlled addition of alkali (25); so here again the inhibitory action cannot be attributed to acidity although the low pH of uncontrolled cultures may contribute to the effect. The third group of enzymes comprises those whose activity is increased by the presence of glucose, other than by a pH effect. It will be noted from table 1 that the only enzyme so far found to belong to this group is glucozymase, so that the increase of glucozymase activity produced by the presence of glucose in the growth medium would appear to be a true adaptation (88).

Kocholaty and Hoogerheide (64) investigating the effect of the presence of glucose in the growth medium on the dehydrogenases of *Clostridium sporogenes*,

claim that glucose increases the activity of the glucose and alcohol enzymes, decreases that of the formic enzyme while the activities of the alanine, asparagine and tryptophan dehydrogenases are unaffected. The pH optimum of proteases excreted by *C. histolyticum* is altered from 7 to 6 by the presence of glucose in the medium (65), but as there may be more than one proteolytic enzyme involved (96) this effect may not be due to an alteration of the pH of optimum activity

TABLE 1

Potential activities of E. coli when grown in media adjusted to pH 7 and 5, and containing glucose (25)

ENZYME	Q UNIT	POTENTIAL ACTIVITY WHEN GROWN		
		(a) At pH 7	(b) At pH 5	(c) In glucose
No glucose effect				
Group I				
Hydrogenase	MB	240	136	146
Catalase	O₂	4200	6360	6310
Arginine decarboxylase	CO₂	2	338	272
Lysine decarboxylase	CO₂	53	194	198
Histidine decarboxylase	CO₂	3	26	33
Glucose effect inhibitory				
Group II				
Ornithine decarboxylase	CO₂	47	960	48
Alanine deaminase	NH₃	32	4	1.7
Glutamic acid deaminase	NH₃	12	3	1.2
Aspartase	NH₃	127	247	15
Serine deaminase	NH₃	855	656	167
"Tryptophanase"	Indole	5.4	1.6	0.2
Alcohol dehydrogenase	MB	52	179	44
Succinic dehydrogenase	MB	43	23	9
Formic dehydrogenase	MB	110	138	58
Formic hydrogenlyase	H₂	75	200	139
Glucose effect stimulatory				
Group III				
Glucozymase	Glucose	38	31	77

Final pH in glucose medium after growth = 5.2 (mean for all cultures).
Potential activities estimated in each case at the optimal pH for the activity of the enzyme concerned.

of one enzyme but due rather to different proportions of different proteases caused by the presence or absence of glucose.

Tryptophan is decomposed aerobically by *E. coli* with the formation of indole. This has been established by many workers (44, 51, 72, 102). Happold and Hoyle have made a non-viable preparation of *E. coli* which will carry out the reaction (44); and they have found (45) that such preparations made from cul-

tures grown in the presence of glucose contain no "tryptophanase" activity while active preparations, from cultures grown in the absence of glucose, are unaffected by the addition of glucose during the experimental test. Fildes (27), investigating the production of "tryptophanase" by *E. coli*, has produced evidence that it is an adaptive enzyme but that cells grown in the absence of tryptophan still possess a small constitutive enzymic activity. He showed further that growth in glucose inhibits the formation of the adaptive portion of the enzyme but has no effect on the constitutive portion. Evans, *et al.* (23) claim that Fildes' constitutive "tryptophanase" is an artefact due to neo-enzyme formation during washed suspension experiments and that, if toluene is present to prevent neo-enzyme formation, then "tryptophanase" is strictly adaptive and shows no constitutive portion. They confirm, however, that *E. coli* grown in a synthetic medium with glucose, as used by Fildes, does possess a small "tryptophanase" activity but that this residual activity is completely abolished if the medium contains, in addition to glucose, phenylalanine or tyrosine in amount slightly greater than the amount of tryptophan present. "Tryptophanase" must consist of a series of enzymes: which particular part or parts of the system is affected by glucose is not known as yet. Krebs (67) has announced that indole is formed by *E. coli* from *o*-aminophenylethylalcohol which may therefore be an intermediate in the formation of indole from tryptophan, but it is not stated how the presence of glucose in the growth medium affects the production of *o*-aminophenylethylalcohol or of *o*-aminophenylacetaldehyde from tryptophan. Reference to table 1 shows that the inhibition of "tryptophanase" activity by glucose is apparently greater than can be explained by a pH effect; this point has been investigated in some detail by Evans, Happold and their co-workers (24) and they find that the formation of "tryptophanase" is inhibited by the presence of arabinose, lactose, glucose, fructose, mannitol and potassium *d*-gluconate, all of which produce acid, but that the presence of *d*-ribose, rhamnose, glucosamine, xylose, sorbitol, galactose and mannose does not have any marked inhibitory effect although the acid production is normal in these cases. Consequently these workers also reject the suggestion that the "tryptophanase" is rendered inoperative by acidity from fermentation.

It has long been the custom to include glucose in the medium for the production of toxin by *Clostridium welchii*. High concentrations of glucose lead to the formation of histamine in the medium (21, 63, 33) while concentrations of the order of 0.5 per cent appear to have a toxigenic effect. The part played by glucose has been investigated by Gale and van Heyningen (39), and they find that glucose markedly increases the production of both α and θ toxins by *C. welchii*, type A, and that the effect cannot be attributed to acidity from fermentation. The pH for optimum θ toxin production in the absence of glucose is 7.5; the addition of 0.45 per cent glucose doubles or trebles the yield of toxin per mg dry weight of organism and, if the pH is controlled throughout the growth period, shifts the pH in the medium for optimum toxin production to 8.0. Alpha toxin is formed only while the pH of the medium lies between 5.5 and 6.5 if no glucose is present, but if 0.45 per cent glucose is added then α toxin is formed at

any pH level between 5.5 and 8.0, with optimum production at 7.0 to 7.5. In neither case can the toxigenic action be related to any possible pH change due to fermentation of the glucose.

On the production of enzymes concerned with the breakdown of proteins and amino-acids (other than by decarboxylation), the presence of glucose has invariably proved inhibitory (29, 37, 25, 89); so that it is interesting to find that Hills (53) has not been able to show any such effect on the production of arginine dihydrolase in pathogenic cocci, especially as this enzyme would seem to be the only known mechanism used by certain cocci to liberate ammonia from amino-acids.

From the various researches outlined here it is clear that, except in cases such as those listed in the first group in table 1, we can discard the idea that the effect of the presence of glucose during growth can necessarily be related to the action of the acid of fermentation. The one weak place in the argument lies in the fact that any pH change produced by fermentation must concern primarily the internal environment of the cell, whereas any pH measurements in the medium are necessarily restricted at present to the external environment. Even so, the action of glucose in inhibiting the formation of enzymes such as the alcohol and formic dehydrogenases (table 1) which are stimulated by acid growth conditions, cannot be explained in any such manner.

Evans, Happold and their co-workers have produced evidence that the presence of glucose results in an increased storage of polysaccharide within the bacterial cell and that this storage is further increased by the presence of phenylalanine or tyrosine in the medium (14). As already mentioned, these workers have shown that the inhibitory effect of glucose on the formation of "tryptophanase" is not complete unless one of these amino-acids is present in addition to the glucose during growth (23). If cells are grown in the presence of glucose and phenylalanine, then they possess no "tryptophanase" but if washed cells of this nature are suspended in the presence of tryptophan under suitable conditions then the power to produce indole is slowly acquired but there is a definite lag period before such indole production begins. It is suggested (24) that this inhibition of indole production or of "neo-tryptophanase" formation is maintained by the metabolism of the carbohydrate stored within the cell during growth in glucose. This hypothesis is supported by the finding that the presence of M/33 iodoacetate increases the lag phase from 30 to more than 120 min. while the presence of M/200 phloridzin or M/50 sodium fluoride also causes slight delay in the recovery of "tryptophanase" activity by the cells. Of the various inhibitors of glycolysis tested, iodoacetate has proved the most effective in prolonging this lag period, and this inhibitor is without significant action upon "tryptophanase" itself. This suggests that metabolism of the stored carbohydrate as far as the triosephosphate→phosphoglycerate stage has an inhibitory action on "tryptophanase" formation. Further it is known that, in muscle, glyceraldehyde inhibits glycolysis at the stage glycogen→Cori ester, and Evans, et al. (24) find that M/100 glyceraldehyde had a marked delaying effect upon the recovery of "tryptophanase" by glucose-grown cells. They find also that the

recovery is assisted by the presence of K ions but not of Na ions, and this fact they think indicates that recovery of "tryptophanase" activity is preceded by metabolism of the stored carbohydrate, since Pulver and Verzar (77) have shown in yeast, and Leibowitz and Kupermintz in *E. coli* (68), that fermentation of glucose is accompanied by a removal of K ions from the medium during the early stages and a return to the initial conditions during the later stages. In these cases the period of maximum K absorption coincides with the period of maximum polysaccharide accumulation within the cells. Conway (10) explains the migration of K ions as follows. The cell membrane is permeable to K or smaller ions but impermeable to Na or larger ions, permeable to phosphate but not to phosphate esters of glucose, adenosine, *etc.* When glucose enters the cell, it is esterified and the effective product of K and phosphate ions within the cell falls so that both ions then enter the cell and the process continues. As the fermentation proceeds later, the esters break down with the liberation of CO_2, alcohol, phosphate, *etc.*, and the effective product of K and phosphate ions again increases and K leaves the cell. If this explanation is the correct one, then the migration of K ions is a consequence of the carbohydrate metabolism and not, as Evans, *et al.* would appear to suggest, a controlling factor. Whatever may be the mechanism, there is considerable evidence that growth in glucose results in a transient accumulation of polysaccharide within the cell. This immediately gives rise to the question of whether the enzyme variations discussed are due to alterations in the permeability of the cell membrane under the influence of the new cellular constitution. This would appear not to be the case for "tryptophanase" since Happold and Hoyle (44) have confirmed their results with toluene-treated organisms where, presumably, the cell membranes will have been impaired; but it should be noted that the recovery of "tryptophanase" activity by glucose-grown cells when suspended in tryptophan takes place too quickly to allow of any significant degree of cell division. In fact, the criticisms levelled by Evans, *et al.* (23) at the investigations of Fildes (27) are based upon the speed with which washed suspensions of cells grown in glucose will acquire "tryptophanase" activity unless a cell-poison such as toluene is added to prevent such neo-enzyme formation. Although a somewhat similar instance of adaptation to substrate without significant growth has been noted for formic hydrogenlyase (91) this would occur only in the presence of nutrient broth, and it would seem that the absolute nature of the inhibition of "tryptophanase" formation by growth in glucose should be carefully reinvestigated and the possibility, that the enzyme is formed equally well in the presence of glucose but that its reaction with its substrate is impeded by polysaccharide accumulation, should be disproved. The solution of this particular problem will obviously depend upon the quantitative extraction of the pure enzymes from the cells and the study of their kinetics *in vitro*.

3. Suboptimal supply of growth factors

Hemophilus parainfluenzae will not grow in broth unless two factors, known as "X" and "V", are added to the medium. The X factor can be supplied by

hemoglobin while the V factor is present in yeast (26, 95). Lwoff and Lwoff (70) showed that the V factor can be replaced in all respects by either coenzyme I or II but not by nicotinic acid or amide, so that the synthetic disability relates to the complete coenzyme molecule. The organism will grow in the presence of suboptimal amounts of the V factor, but its ability to oxidize certain substrates is then impaired. The activity of the washed suspensions towards these substrates can be brought up to normal by the addition of sufficient coenzyme to saturate the enzymes concerned. It appears that, during growth in the presence of suboptimal amounts of coenzyme, the coenzyme available is distributed among the total crop of cells, all of which then show a deficiency in those activities requiring the presence of coenzyme. The X factor is replaceable by hemoglobin or hematin so that it appears probable that this factor supplies some hematin compound necessary for respiration, as some strains can dispense with it for anaerobic growth (2, 22, 66). Since Keilin and Hartree (58, 59) have shown that catalase is a hematin compound, it has been suggested that the X factor is involved in the synthesis of catalase, and this idea is supported by the finding (85) that hematin can be replaced by cysteine; in the presence of cysteine, hydrogen peroxide would be reduced and catalase rendered unnecessary. In this manner the probable functions of the X and V factors have been elucidated. It is possible that other growth factors may act in similar ways by providing coenzymes or prosthetic groups of enzymes essential to the existence of the organism. Thus, Hills (52) has shown that *Staphylococcus aureus* grown in the presence of suboptimal amounts of thiamine, which is a growth substance for this organism, oxidizes and dismutes pyruvic acid at a rate slower than that of the same organism grown in the presence of optimal quantities of the growth factor. The addition of thiamine to the washed suspension is again able to make good the deficiency. It is probable that the active agent is cocarboxylase (thiamine diphosphate) which the organism is able to form by phosphorylation (3). Silverman and Werkman (83) have shown similarly that *Propionibacterium pentosaceum* is unable to synthesize thiamine but that exacting strains can be trained to carry out the synthesis. The rate of anaerobic pyruvic acid metabolism, in the presence and absence of added thiamine, can be used as a measure of the thiamine synthesized. Quastel and Webley (79) have shown that the oxidation of acetic acid by propionic acid bacteria is also increased by the addition of thiamine to washed suspensions.

There is a large assortment of bacterial growth factors now known (87), although a function has as yet been attributed to but a few of them. Deficiencies of any of these in the medium used for the growth of an exacting organism may result in decreased activity of some enzymes and it seems that, in the case of an exacting organism such as *C. acetobutylicum* (11), the growth factors must also be added to the suspending phase of "washed" suspensions if the organisms are to retain certain activities after the preparative treatment; this will be dealt with in detail later.

These factors become obvious when dealing with organisms for which the coenzymes are growth factors, *i.e.*, in those cases where there is no power to

synthesize the coenzyme in the cell. It is possible that non-exacting organisms are unable to synthesize sufficient coenzyme to saturate their enzymes in certain media. Thus, *E. coli* possesses a malic dehydrogenase requiring the presence of a coenzyme identical with the coenzyme I of animal tissues, but washed suspensions prepared from cultures grown in casein digest show a considerably enhanced malic dehydrogenase activity if coenzyme I is added to the suspending water (38). The degree of saturation of the enzyme with coenzyme is found to vary considerably with the nature of the culture medium, being very low on a salt-lactate medium, 20 to 30 per cent on a tryptic digest of casein, and rising to 70 per cent if cozymase is added to the digest. Addition of nicotinic acid or amide, adenylic acid, *etc.*, to the medium all raise the coenzyme saturation of the malic dehydrogenase, but none of these parts are equal in efficiency to the complete coenzyme molecule. These results indicate that the organism is able to synthesize sufficient coenzyme for growth purposes but not necessarily sufficient to saturate the enzymes needing coenzyme. If the production of enzyme and coenzyme are followed with the age of culture, it is found that cultures in the early logarithmic phase of growth have little enzyme or coenzyme, that synthesis of both enzyme and coenzyme proceeds as growth continues but that the synthesis of coenzyme falls off before that of enzyme so that there is an apparent decrease of activity before growth ceases. These effects cannot be obtained with the alcohol dehydrogenase of *E. coli* (94).

If washed suspensions of *E. coli* are boiled, their coenzyme I is quantitatively liberated into the suspending water (38). In a similar fashion, the addition of a suspension of boiled organisms, or the supernatant fluid therefrom, acts as a good source of unknown coenzymes and can be added to active suspensions as a test for coenzyme saturation with regard to the activity under test. This method has been used to demonstrate the existence of a coenzyme for aspartase II (29) in *E. coli*, for certain dehydrogenases in *E. coli* (107, 108), and for the decomposition of *dl*-serine by *C. welchii* (103). In this last case, the method was used to show the synthesis of both enzyme and coenzyme during the development of growing cultures. If there is reason to suspect that a given activity may be showing variation as a result of suboptimal supply or synthesis of coenzyme, it is thus possible to test the hypothesis by the addition of a thick boiled suspension of organism to the experimental suspension and, if positive results are obtained, fractionation of the boiled suspension can be carried out so that a simpler fraction can be added to the growth medium in later experiments to ensure that the organism will be supplied with optimal amounts of coenzyme. Even when excess coenzyme is supplied in the growth medium, the organism is sometimes unable to assimilate or utilize more than will give an enzyme-coenzyme saturation of approximately 70 per cent (38).

VARIATIONS DUE TO PHYSICAL FACTORS INVOLVED DURING GROWTH

1. Oxygen tension

A facultative anaerobe such as *E. coli* possesses potential enzyme mechanisms enabling it to metabolize under atmospheres ranging from nil to 100 per cent

oxygen. Aerobically, the organism obtains most of its energy by oxidative reactions, but it is doubtful whether these enzymes can act anaerobically although some take part in mutual oxidation-reduction reactions. Consequently the organism needs to develop a different enzyme constitution for anaerobic existence from that suitable for aerobic existence. Stephenson and Gale (89) studied this point with regard to the deaminases of *E. coli*, and they found that the oxidative deaminases attacking glycine and alanine are formed best when growth occurs aerobically and that anaerobic growth conditions inhibit their formation. On the other hand the anaerobic deaminase systems attacking *dl*-serine and *l*-aspartic acid (37, 29) are markedly stimulated by conditions of anaerobic growth; thus it appears that anaerobic growth conditions stimulate the formation of anaerobic mechanisms and inhibit the formation of aerobic enzymes. The activity of washed suspensions towards serine and aspartic acid falls off on standing in air, and this loss of activity is due, in part, to an oxidation process, as it can be prevented by the presence of certain reducing agents such as glutathione (GSH). It is now known that many enzymes are active only *when the enzyme substance is in a reduced state (46, 54)*, and it has been suggested that such enzymes depend for their activity upon the presence of free thiol groups in the enzyme molecule.

The strictly adaptive enzyme, formic hydrogenlyase, is inhibited by strongly aerobic conditions during growth so that aeration of the medium or growth on the surface of agar results in inactive organisms (86, 106). Formic hydrogenlyase is an anaerobic mechanism which is produced adaptively by *E. coli* under growth conditions in which the formic dehydrogenase is ineffective. Formic dehydrogenase reacts with oxygen through the cytochrome system of the organism (30) and is an exception to the usual rule in that increasing oxygen tension during growth results in decreased dehydrogenase activity of the organism. This seems to be due to the fact that the enzyme itself is capable of reversible oxidation in the presence of the substrate and that the oxidised form is inactive. In the presence of formate and oxygen, two reactions take place. The first is an oxidation of the formate to water and carbon dioxide, the oxygen being carried by the cytochrome system; and the second is a direct oxidation of the enzyme substance resulting in inactivation. Under normal conditions in the presence of the organism's normal complement of cytochrome, the oxidation of the substrate is the predominating reaction and the inactivation takes place slowly if at all. If however the cytochrome is removed from the enzyme preparation by digestion, then the oxidation of the enzyme substance takes place much more rapidly until in the limiting case when all the cytochrome has been removed, the enzyme is inactivated so rapidly that no significant oxidation of the substrate occurs. The enzyme can be "protected" from inactivation by the presence of high concentrations of methylene blue or cytochrome or other oxygen carriers which promote the oxidation of the substrate and, conversely, in the presence of a constant amount of carrier the rate of inactivation of the enzyme depends upon the oxygen tension, the higher the tension the more rapid the inactivation. The results are similar to those that might be expected if hydrogen peroxide

were formed during oxidation of the substrate, but all the usual tests failed to detect peroxide formation. These results were obtained mainly with cell-free preparations of the dehydrogenase, but they probably explain the decreasing formic dehydrogenase activity of cultures grown in increasing oxygen tensions, as no evidence could be obtained to show that the cytochrome content of the cells is affected by the oxygen tension holding during growth. The effect has been studied with this enzyme only, but it may well play a part in the sensitivity to oxygen displayed by the strictly anaerobic bacteria which do not contain cytochrome or other carrier.

A further peculiar conditioning of enzyme constitution by oxygen is found in the oxidation of tyramine by *E. coli* and *Pseudomonas pyocyanea* where immediate oxidation will occur only—on mixing the substrate with the washed suspension—if the organisms have been grown in the presence of both tyramine and air. The organism grown anaerobically in the presence of tyramine displays a lag phase after addition of the substrate before oxidation begins. This lag phase does not seem to be associated with any breakdown of the tyramine, and its duration is proportional to the amount of tyramine added. The course of the oxidation by the aerobe appears to follow a different course from that accomplished by the anaerobe (34).

2. The pH of the medium during growth

With few exceptions the importance of the pH of the medium during growth has not been appreciated until recently. It has been suggested in the past that the action of glucose in suppressing the formation of deaminases and proteases is due to the action of the acid produced from it by fermentation and this hypothesis has been discussed fully above. Kocholaty and Hoogerheide (64) claim that alteration in the pH of the medium results in a shift of the pH optimum of certain enzymes in *C. sporogenes* and of the excreted proteases of *C. histolyticum* (65), although in this latter case the "pH change" was produced by the incorporation of glucose in the medium and was not necessarily associated with any one protease enzyme. Gale (31), investigating the action of glucose in promoting the formation of amino-acid decarboxylases by bacteria, showed that the effect could be reproduced by adjusting the pH of the medium before growth to that degree of acidity normally produced by the fermentation of the glucose. The decarboxylases are thus produced by bacteria in response to an acid environment, and the lower the pH during growth the higher are the decarboxylase activities of the resulting organisms. It has been suggested (43) that these enzymes are in effect mechanisms produced by the organisms to counteract an environment which is becoming too acid for continued existence, since the decarboxylation of an amino-acid results in the formation of alkaline amines.

Silverman and Werkman (84) have shown that there are two mechanisms in *Aerobacter aerogenes* which decompose pyruvic acid. These are (a) a hydroclastic breakdown to acetic and formic acids, and (b) the condensation of two molecules of pyruvic acid to acetylmethylcarbinol and CO_2. The enzyme ("carbinol

enzyme") responsible for the latter reaction is optimally active at an acid pH of the order of 5 and, like the amino-acid decarboxylases which also have very acid pH optima, is formed only when the organism grows under acid conditions. If the growth medium is controlled at an alkaline pH during growth, the production of the "carbinol enzyme" is suppressed and acetylmethylcarbinol no longer formed. It is not possible to "train out" this enzyme by serial subcultivation in alkaline media, as it is formed immediately cultivation is resumed in acid media. It can be obtained in a cell-free condition by grinding the cells with particles of glass; and confirmation can be obtained in this way that this enzyme is formed by the cell only in response to acid growth conditions.

Following these observations, Gale and Epps (35) have made a study of the general effect of growth pH upon enzyme constitution and metabolism of bacteria. They found that E. coli can grow in media adjusted to pH values anywhere between 4.5 and 9 but that the metabolic activities of the organisms during growth tend to shift the pH of the medium towards neutrality. If this drift in the external environmental pH is checked by buffering the media, then pH is found to have very marked effects upon enzymic constitution. To understand the variations which occur, it is necessary to make use of three terms which can be defined as follows:

pH of optimum activity: that pH at which an enzyme in a given washed suspension displays its highest activity. Whereas Kocholaty and Hoogerheide (64) claim that the value of the pH of optimum activity alters in some cases in *C. sporogenes* with the pH during growth, Gale and Epps (35) were unable to find any such effect with the enzymes they studied in *E. coli* but found that the value is constant for any one enzyme and independent of the pH during growth.

Potential activity: the activity shown by a given washed suspension when that activity is estimated at the pH of optimum activity for the enzyme concerned. The potential activity varies with the pH during growth as described below.

Effective activity: the activity displayed by a given washed suspension when that activity is estimated at an experimental pH equal to that of the medium in which the organisms were grown. The effective activity is thus the activity exerted by the organism in the medium and represents that portion of the maximum activity which is effective in culture.

The enzymes studied in *E. coli* can be divided into two classes according to the nature of their variation with pH during growth:

1. Those enzymes whose potential activity increases as the pH during growth deviates from the pH of optimum activity, the loss in activity of each enzyme unit caused by the deviation from the pH of optimum activity being compensated by an increased enzyme formation so that the effective activity is approximately constant throughout the pH range of growth.

2. Those enzymes whose formation in the cell is greatest when the pH during growth approximates to their pH of optimum activity. In some cases where the pH of optimum activity lies near neutrality, there is a restricted degree

of compensation over the centre of the growth range, so that the effective activity is approximately constant for pH values of 6 to 8 during growth, but falls off rapidly outside this range.

Of the enzymes investigated in *E. coli* and *M. lysodeikticus* the following fall in group 1: formic dehydrogenase, alcohol dehydrogenase, formic hydrogenlyase, urease, catalase, and fumarase.   In these cases the potential activity is smallest in cells which have been grown at the pH of optimum activity, and increases as the pH during growth deviates from this value.   If the effective activity is estimated in each case, it is found that this is approximately constant for all cultures so that the organism has so adapted its enzyme constitution that its activity towards the substrates of these enzymes is independent of pH during growth (see fig. 8, ref. 35).   Formic hydrogenlyase is an exception in that its minimum potential activity does not fall at pH 6.5 (approx.) the value equivalent to its pH of optimum activity; in this case, we find that the addition of formate to the medium to evoke the enzyme has a toxic effect, so that growth will not take place at pH values acid to 6.3.   The potential activity is least for cultures grown at pH 7.5; for values above this, the usual compensatory formation occurs but for values acid to this there is a very rapid increase in both potential and effective activities, so that the highest activities occur when growth takes place at the acid limit of formate tolerance (see fig. 10, ref. 35).   Thus, it seems in this case as though the organism produces the enzyme most effectively where the substrate appears to be most toxic so that the formic hydrogenlyase enzyme can be regarded as a protective mechanism.   Since hydrogen peroxide, urea and alcohol are also known to be toxic to bacterial growth, it would seem as though the enzymes in group 1 may be classed as detoxication or general protective mechanisms.   The position of fumarase is not so certain, as there is little evidence at present that fumaric acid is toxic to bacteria, although it is difficult to interpret the action of such substances when the organisms have evolved efficient mechanisms for their removal.   Thus, alcohol is toxic only in comparatively high concentrations and formate only when the dehydrogenase is inactivated by anaerobic conditions and at acid pH values.   That these variations in group 1 are due to actual alterations in enzyme content of the cell has been shown by investigation of the catalase, urease and fumarase activities before and after lysis (with lysozyme) in *M. lysodeikticus*.   Both intact cells and lysed preparations show exactly the same type of activity variations with pH during growth.

The enzymes falling into group 2 for these two organisms are: hydrogenase, succinic dehydrogenase, glucozymase, "tryptophanase", aspartase, the decarboxylases of arginine, ornithine, lysine, and histidine, and the deaminases of alanine, serine, and glutamic acid.   The first three in the list show restricted compensatory formation over the centre of the growth range, thus hydrogenase which has its pH of optimum activity at 6.0 displays the highest potential activity in cultures grown at pH 8.0, so that the hydrogenase activity is approximately constant for cultures grown at pH 6.0 to 8.0 but falls very rapidly outside those limits (see fig. 11, ref. 35).   The rest of the enzymes in this group

are concerned with the decomposition of amino-acids and, in most cases, have pH values of optimum activity removed from neutrality. The decarboxylases have pH values of optimum activity ranging from 4.0 to 5.5 (external environment of the intact cell), and they are formed in the cell only when growth takes place at pH values approaching their pH optima. That this is due to an actual variation in enzyme content of the cells has been shown in the case of lysine decarboxylase where the enzyme survives disintegration of the cell by acetone treatment and can be extracted into solution (36). It is not possible to state definitely the pH values of optimum activity for the deaminases of *dl*-alanine, *dl*-serine and *l*-glutamic acid, as the value appears to depend in each case on the nature of the buffer in which it is determined. Thus, these three enzymes all display a peak of activity for pH values between 7.0 and 7.5 in phosphate buffer and between 8.5 and 10 in borate or veronal buffers (35). Kocholaty and Hoogerheide (64) find that the alanine dehydrogenase of *C. sporogenes* displays two peaks of activity when the range 6 to 10 is covered by phosphate "buffers" and the activity estimated by methylene blue reduction. When the variation of potential activity with pH of growth is investigated, it is found (35) in these three cases that little deaminase activity is displayed by cultures grown under acid conditions and that the activity becomes greater the more alkaline the growth medium. In the case of glutamic acid the increase in activity from pH 5 to 9 is linear; with alanine the activity is steady for growths at pH 5.5 to 7.5 and then rises steeply to the alkaline limit of growth; and with serine the activity of cultures shows two peaks, one for cells grown at pH 7.5 and a higher peak for cells grown at pH 8.5. There is evidence then that the enzyme is formed in response to an alkaline medium, but that in some cases a stimulation of the formation takes place when the pH during growth is of the order of the pH of optimum activity as determined in phosphate buffer. The amino-acid enzymes can thus be regarded as complementary systems: the decarboxylases being produced in response to an acid environment and tending by their activity to neutralize that acidity, and the deaminases being produced in response to an alkaline environment and tending to neutralize the alkalinity since their action leads to the evolution of ammonia and formation of acid products. The aspartase enzyme of *E. coli* differs from the other deaminases studied in that its formation is inhibited by strongly acid or strongly alkaline conditions and is greatest when growth occurs between pH 5 and 6. The neutralizing function carried out by the amino-acid decarboxylases in *E. coli* may be undertaken by other systems in organisms which do not possess such enzymes in their potential enzymic constitution: thus *C. acetobutylicum* which does not possess any amino-acid decarboxylases (33), produces when grown under acid conditions, enzymes bringing about the reduction of acetic and butyric acids to acetone and butyl alcohol, respectively (11). Again, in this case, the formation of the enzymes within the cell is followed by a rise in the pH of the medium during uncontrolled culture.

Further evidence that these variations with pH during growth are due to alterations in enzyme formation has been produced by extension of the investi-

gations to the production of toxins by *C. welchii* (39). *C. welchii*, when growing in a suitable medium, excretes into the medium two toxins (97, 98), proteolytic enzymes, and hyaluronidase (74). The alpha toxin has been studied in detail by Macfarlane and Knight (71) and found to be closely associated with and probably identical with lecithinase, but the specific substrate of the theta toxin has not as yet been identified. Hyaluronidase and the toxins are extracellular enzymes whose activities can be estimated *in vitro* with accuracy (97, 74, 98, 39), and any variations in their production will not be complicated by permeability considerations. Although no activity-pH curves have been published for these enzymes, when the toxin production per mg dry weight of organism is studied, the curves obtained for both α and θ toxins display a group 2 type of variation with pH during growth whether glucose is incorporated (pH controlled throughout fermentation) or not within the medium. On a similar basis hyaluronidase displays what is probably a group 1 variation showing least formation per mg dry weight of organism when growth occurs at pH 7.

3. Temperature during growth

The effect of temperature during growth upon the enzymic activities of bacteria has seldom been investigated. Eggerth (21) found that the temperature of cultivation can markedly affect the production of histamine by various bacterial species; some strains of *E. coli* produced more histamine when grown at 20 to 30 C than at 37 C, while others were more active at higher growth temperatures. Organisms such as *C. welchii* produce more histamine at high temperatures than at low ones. These effects have been confirmed with washed suspensions of *E. coli* (31) where it is found in general that the amino-acid decarboxylases are formed more prolifically at 27 C than at 37 C. The effect may be due to unusual thermolability of the enzymes concerned, but this is unlikely as the effect differs from strain to strain and is characteristic of a particular organism rather than of any one enzyme.

FACTORS APPARENTLY RELATED TO THE ORGANISM ITSELF

1. Age of the culture

Wooldridge and his co-workers (104) pointed out that, when studied by the washed suspension technique, the activities of certain dehydrogenases of *E. coli* appear to vary with the age of the culture from inoculation to harvesting. In general, "young" cultures have little activity, but this increases as the growth continues and later diminishes again as the culture ages. The variation was not constant for all enzymes, being least for formic dehydrogenase of the enzymes studied. They showed further (105) that the effect is not due to the size or viability of the cells in the suspensions but appeared to be associated with the phase of growth at the time of harvesting. These workers postulated that the organisms are most active during the logarithmic phase of growth when the "cell is in a state of structural fluctuation." The variation of enzymic activity with the age of the culture has since been studied with many enzymes and may be very marked in some cases such as the serine deaminase system of *E.*

coli (37), where the Q_N rises from 200 for a 4-hr culture to 1100 for a 10 to 11-hr culture and then falls again to 200 by the end of 24 hours. In the experience of the author, the maximum activity is usually shown by the culture taken at the time when active cell division has just ceased as judged by the stabilization of the turbidity and not, as suggested by Wooldridge, during the logarithmic phase. In many cases studied (29, 31 to 33, 38, 39, 103), cultures taken at the beginning of the logarithmic phase have little enzyme activity, this activity then increases linearly during the logarithmic phase, and once the initial growth has ceased and before it starts again on autolysis products, the activity may remain steady or, more usually, may decrease slowly,—although there are cases such as that of serine deaminase mentioned above where this loss of activity may be very rapid. That the variation with age of culture is a real variation and not merely a matter of changing permeability has been shown in the cases of the extracellular proteases of *C. histolyticum* (96), of hyaluronidase and of α and θ toxins of *C. welchii* (39) where the variation of enzyme production per mg dry weight of organism with age of culture is just such as described. In the case of the protease excretion by *C. histolyticum*, there is a further appearance of enzyme in the medium after the cessation of growth but this new enzyme differs from the bulk of the protease excreted during growth and is probably endoenous protease liberated by autolysis of dead cells (96).

In the majority of cases the effect is probably not due to any one cause as, addition to the factors already discussed, the following may play a part:

a. Varied rates of synthesis of enzyme with growth phase.

b. Varied rates of synthesis of coenzyme with growth phase.

c. Removal of toxic substances or inhibitors from the growth medium.

d. Exhaustion of growth factors in the later stages of growth.

e. Alteration of pH of medium during growth.

f. Autolysis, proteolysis, *etc.*, of cells and enzymes in old cultures.

g. Varying degree of cell permeability towards substrate.

At the present state of our knowledge it is not possible to say how many of these factors are involved in any one case, and the most satisfactory approach at the moment is to outline examples in which these factors have been demonstrated to play a part. It is perhaps of significance that all the enzymes studied in this respect would appear to have a catabolic function, and it may be that "young" cultures though possessing little activity with regard to these enzymes may possess highly active synthetic systems.

In the case of malic dehydrogenase of *E. coli*, the activity shows an unusual variation with age of culture in that the activity reaches a maximum early in the growth phase and begins to fall off before growth ceases (38). It has been shown above that in this case the synthesis of coenzyme I lags behind that of enzyme and so produces this effect, so that if suspensions are saturated with cozymase during test then the more usual type of variation, reaching a maximum at the end of active growth, is obtained. Woods and Trim (103), investigating the variation with age of culture of serine deaminase of *C. welchii*, added thick

suspensions of boiled organisms as a source of coenzyme and were able to show that "young" cultures are deficient in both enzyme and coenzyme so far as can be determined within the restrictions of the washed suspension technique.

Aspartase of *E. coli* shows a typical variation with the age of the culture. In order to determine whether the small activity of young cultures is an effect of age alone or due to some interaction with the medium, Gale (29) grew a culture until the aspartase activity was well developed, removed the organism and resterilized the medium by filtration through a Seitz filter. The resterilized and semi-exhausted medium was then inoculated again with *E. coli* and incubated until a turbidity was first visible and a washed suspension could be prepared. The activity of this first "young" culture was then determined and proved to be equal to that of a normal culture at the end of the growth phase. Thus, in this case, the small activity of normal "young" cultures cannot be attributed to an effect of age alone but would seem to be dependent upon some chemical change in the medium brought about by the metabolic activities of the organism. Whether this is the removal of a toxic substance, or the synthesis of an essential enzyme factor is not known.

The amino-acid decarboxylases of *E. coli*, and species of *Proteus* and *Clostridium*, etc., all have a small activity in "young" cultures and the activity develops to a maximum by the end of growth (31, 33). In such cases, however, the enzymes are produced in response to an acid environment, and when the organisms are growing in a medium containing fermentable sugar, the appearance of the enzymes is conditioned by the production of a favorable acidity. Thus, the histidine decarboxylase of *C. welchii*, which has a pH of optimum activity lying between 2.5 and 3.0, is not formed when growth occurs in the presence of glucose until the pH of the medium has fallen below 5 (33). In a similar way, the acetoacetic acid decarboxylase of *C. acetobutylicum* does not appear in cells harvested from the medium until the pH has fallen to about 4.5 (12, 13) while hydrogenase activity appears only in the early stages of growth when the pH of the medium is not strongly acid. These findings are correlated with the pH values of optimum activity for the two enzymes, the former being 4 and the latter about 8. Thus, the metabolism of the cells growing in a fermentable medium will differ greatly according to the age of the culture at the time of harvesting: "young" cells, harvested before the pH of the medium has dropped below 5.5, are unable to produce acetone or butyl alcohol from glucose but may have hydrogenase activity, while "ripe" cells harvested from later cultures, in which the production of acetone has started and when the pH is at a minimum, possess no hydrogenase activity but a very active acetoacetic acid decarboxylase.

Kocholaty and Hoogerheide (64) have reported an interesting effect of glucose on the influence of age in the dehydrogenases of *C. histolyticum*. They find that, in general, the dehydrogenase activities estimated at 20, 45 and 70 hr after inoculation steadily decrease, with the exception of alcohol dehydrogenase whose activity increases over this period; if, however, growth takes place in the presence of glucose, then the alcohol dehydrogenase activity falls off rapidly in the

aging culture. Since alcohol dehydrogenase has optimum activity at pH 8.0, this effect of glucose may be a pH effect on the enzyme by the acids of fermentation.

With the exception of the extracellular enzyme variations mentioned above, these effects due to age of culture have been investigated in washed suspensions, and it has yet to be proved that they represent true variations of enzyme content with time and not variations in enzyme-substrate accessibility. The general conclusion from all the results would seem to be that the age of the culture may have marked effects upon the enzyme constitution of bacteria and that in studies of bacterial enzymes (catabolic ?), it is usually most satisfactory to harvest organisms as near as possible to the end of the period of active growth to avoid complications due to this factor.

The results so far discussed have concerned the variation of separate enzyme systems. Winslow and Walker (101) have collected a considerable volume of work dealing with the variation with the age of the culture of general metabolic activity studied by means of the respiratory oxygen consumption, CO_2 production, etc., and the results appear to show that the highest activity occurs during the early logarithmic phase and falls off again before the end of the period of active growth,—when washed suspension and enzyme studies show that many enzymes are at their maximum activity as expressed on a basis of dry weight of organism. It must be remembered first that "over-all" studies on organisms growing in culture are dependent upon factors such as the exhaustion of substrates in the medium and consequently do not necessarily give accurate indication of the enzymic activities of the organisms (49). Further the activities in much of the work quoted by Winslow and Walker are expressed per cell and are consequently vitiated to the extent that there are alterations in the size of the cell. Hershey (47) has pointed out that the high activities obtained per cell in "young" cultures are an artefact due to neglect of the change of size occurring in the cells of a freshly inoculated culture. When bacterial numbers (viable count) and cell nitrogen are plotted logarithmically against time, the former gives a typical "growth curve" showing a lag phase merging into a phase of logarithmic increase while the latter shows a steady increase from the time of inoculation until growth begins to fall off after 6 hours or so. The lag phase is thus due to the cells increasing in size without division during the early stages of growth. Further, if media are seeded from young and old cultures, the rate of growth as measured by turbidity or cell-nitrogen is the same for both inocula, but if measured by cell numbers then there is a much longer lag phase, or decrease of multiplication rate, in the culture seeded from the old inoculum (48). These factors naturally affect the "age of culture curves;" Hershey and Bronfenbrenner (50) showed that if oxygen uptake is plotted against viable count then the usual high peak of activity is obtained during the early logarithmic phase but if the results are plotted against cell-N then a straight line up to 24 hours is obtained. Thus the period of "physiological youth" is an artefact. Clifton (8) has also carried out a series of experiments which show that the metabolic activities of bacteria are influenced by their size and changing en-

vironment and not by the age of the culture. The enzyme studies reviewed here have shown what a diversity of factors can influence the bacterial physiology and most of these factors are continually changing in the medium of a growing culture. It seems highly probable that each cell in the growing culture has an enzymic constitution determined by the environmental conditions holding at the moment of its division, and that the effects due to "age of culture" or "life cycle" (100) represent the results of continuously changing environmental conditions on continuously dividing—and chemically active—cells.

2. Permeability of cell membrane

If the rate of breakdown of a substrate is limited by the rate of diffusion of that substrate through the cell membrane, then it follows that apparent variations in enzyme activity may be due to alterations in the permeability of the membrane. In many cases our present methods of investigation do not as yet permit us to say whether permeability is actually a limiting factor or not, but one case in which it does seem to play an important role is that of the lactase activity of *E. coli mutabile*. Deere (15, 16) has shown that the activity of the white, "non-lactose-fermenting" strain can be increased to that of the red, lactose-fermenting strain by drying the organisms or treating them with acetone, certain antiseptics, *etc.* in such a way as to disintegrate the cell membranes. He interprets these results as meaning that the white strain is inactive towards lactose because the cell membrane is impermeable to this substrate but that if the membrane can be injured so as to become permeable, then the cell displays normal lactose activity.

3. Cell division as a factor in enzyme variation

Many workers have investigated whether variations in enzymic activity can be brought about without division of the cells concerned. Stephenson and Yudkin (92) have shown that yeast, treated with ultraviolet radiation so as to render it non-viable, could still produce the adaptive enzyme galactozymase when suspended in galactose solution; but in *E. coli* adaptation to galactose could only be obtained after cell division in the presence of the substrate (88). Stephenson and Stickland (91) have shown that *E. coli* grown on agar and suspended in formate broth will develop formic hydrogenlyase although total cell counts before and after the adaptation showed that no significant growth had occurred. These examples show that adaptive enzymes can be developed in cultures which have not undergone division, although a certain amount of growth without cell division may have occurred. There is no record of adaptation by completely sterile cultures. Karström (57) concluded that, although cell division is not necessary for enzymic variation to occur, there must be opportunity for the cell to form new cell-substance. In other words, enzymic variation cannot occur unless the cells are in such a state that growth is possible although the growth may not necessarily proceed as far as actual division. This would seem to apply to the majority of enzyme variations discussed here, *e.g.*, suspensions of *E. coli* grown at pH 7 cannot develop amino-acid decarboxylases

if incubated at pH 5 in the presence of the specific amino-acid and buffer only (31), nor will the formic or alcohol dehydrogenase activity increase as it would if growth occurred at pH 5 (35). On the other hand, the recovery of "trypto-phanase" activity by washed suspensions of glucose-grown cells suspended in the presence of tryptophan would appear to involve enzyme formation in the absence of any growth (23).

The preparation of active cell suspensions

The usual method for the preparation of cell suspensions consists of centrifug-ing the cells out of culture, followed by washing and final suspension of the washed cells in distilled water, saline or Ringer's solution. This method suffices for many of the enzyme studies with organisms such as *E. coli* but may not give results with more sensitive enzyme systems or more exacting organisms. In suspensions prepared in this way certain activities may fall off very rapidly, so rapidly in some cases that all activity is lost during the washing process. The causes of this decay of activity include (a) diffusion out of the cell of coenzymes, *etc.*, (b) decomposition of coenzymes, *etc.*, by cell activity, (c) oxidation of the enzyme protein, (d) absence of factors for enzyme maintenance.

The serine deaminase of *E. coli* and of *C. welchii* decays rapidly on standing of the washed suspensions (37, 103), and the loss of activity appears to be ssociated with the diffusion of some coenzyme-like factor out of the cell, as it can be prevented by the addition to the suspending water of boiled organism, *etc.* The similar loss of aspartase activity from *E. coli* (29) can be prevented by the addition of adenosine and other adenylic acid derivatives to the suspending water. The decay of serine deaminase is also associated with an oxidation process as the lost activity can be restored only by the action of a reducing agent such as glutathione (GSH) and phosphate. It is possible that this enzyme requires free -SH groups in its structure to remain active, as suggested for other enzymes (54), and consequently the activity falls as the enzyme is exposed to air. There is considerable loss of serine deaminase activity during the preparation of the washed suspension but this can be checked, to some extent at any rate, by the addition of phosphate and a reducing agent to the washing water. It has also been reported (64) that the dehydrogenases of *C. sporogenes* lose a large part of their activity during the washing of the cells.

A very complex example has recently been studied by Davies and Stephenson (11) who have succeeded in preparing suspensions of *C. acetobutylicum* capable of fermenting glucose and pyruvate. When suspensions of this organism were prepared in the usual fashion, they proved to be completely inactive; washing in freshly boiled phosphate buffer preserved some small activity, but highly active suspensions could be obtained only if all the constituents of the basal growth medium were present,—salts, asparagine, yeast autolysate, *etc.* The cells would then retain their activity for a short time after preparation. Later (12) it was found that the presence of glucose has a marked effect on this loss of activity of cells removed from culture. Glucose not only prevents further loss but even, in some cases, leads to a recovery of activity lost during the prep-

aration of the cells. The glucose is, of course, fermented and its effect lasts only so long as it is present, so that a high concentration is necessary to maintain the activity for any considerable time. It is not possible to state whether glucose itself or one of its breakdown products is responsible for this maintenance but it would appear to be definite that the effect is not due to hydrogen.

DISCUSSION

The enzymic constitution of the bacterial cell can vary over a wide range and is influenced by many factors acting during the growth of the cell. Many of these factors have been described in this review but there are doubtless others as yet to be investigated. The picture we get of the organism is of a cell with many, though limited, potential activities from among which the actual activities of any individual are largely determined by the physical and chemical conditions holding at the moment that that individual divides from its mother-cell. The potential constitution differs from genus to genus, species to species, and strain to strain, and it is by these differences that we separate genus, species, and strain. The potential constitution is characteristic of each of these; the actual constitution is characteristic of the individual cell.

We find that the growth conditions determining the incorporation of a particular enzyme from the potential into the actual constitution vary considerably from enzyme to enzyme. Some enzyme systems require very exacting conditions for their formation, needing the presence of the specific substrate, a pH approximating that of their optimum activity, a suitable growth temperature, and a certain phase of cell-growth before their production is optimal. Thus the amino-acid decarboxylases of *E. coli* are formed optimally only when growth occurs in the presence of the specific amino-acid concerned, at a pH acid to 5, at a temperature below 30 C, and when the culture is approaching the end of the period of active cell-division (31). These enzymes are not formed at an acid pH in the absence of substrate; or at a pH higher than 6.5 in the presence of the substrate; or at a temperature higher than 37 C. On the other hand, the formation of some enzymes seems to take place relatively independently of the growth conditions: thus formic dehydrogenase of *E. coli* is formed constitutively at any temperature, showing the smallest variation with "age of culture" that has been demonstrated for any of the enzymes of this organism (104), and its formation is so controlled that the enzymic activity is effectively constant whatever the pH of the external environment (35). If we allow ourselves to speculate on the nature of these variations, it is possible that they can throw some light on the function of bacterial enzymes and the economy of their formation.

The enzymes with which the bacterial cell is equipped have at least four functions to fulfil: *a*, to release energy for continued existence and division, *b*, to provide essential metabolites and nutrilites, *c*, to detoxicate toxic metabolic products, and *d*, to stabilize the internal environment in a variable external environment. We have shown how the enzymic activities of a cell such as *E. coli* can alter quantitatively and qualitatively in response to alterations in the pH of the external environment (35). Thus in the cell growing in an acid en-

vironment, the production of amino-acid decarboxylases is stimulated while that of the deaminases is suppressed. Similarly when a cell grows in an alkaline environment the formation of the deaminases is stimulated and that of the decarboxylases suppressed. On the one hand an acid environment results in the stimulation of mechanisms producing an alkaline reaction while, on the other hand, an alkaline growth environment results in the metabolism of the cell being swung towards acid-producing systems. That this has its effect on the external medium can be seen in the drift of the pH of media towards neutrality during growth (35), but the effect on the internal environment must be even more marked. We can look upon these amino-acid systems in *E. coli* and on the enzymes bringing about, for example, the reduction of fatty acids to alcohols in *C. acetobutylicum* as mechanisms whereby the organism is enabled to grow in an environment covering a wide pH range since, by their action, the internal environment is stabilized within a smaller pH range than that measured in the external medium.

TABLE 2

pH of optimum activity for amino-acid decarboxylases

SUBSTRATE	ORGANISM	CELL-FREE ENZYME	INTACT CELL	DIFF.
Histidine	*C. welchii*	4.5–5.0	2.5–3.0	2.0
	E. coli	5.5	4.0–4.75	0.75–1.5
Arginine	*E. coli*	5.5	4.0–4.75	0.75–1.5
Lysine	*E. coli*	6.0	4.5–5.0	1.0 –1.5
Tyrosine	*S. faecalis*	5.5	5.0–5.5	0.0 –0.5
Acetoacetic acid	*C. acetobutylicum*	5.5	4.0–5.0	0.5 –1.5

We have not as yet any satisfactory method for measuring the internal pH of the bacterial cell, but indirect evidence that a difference exists between the inside and outside of the cell when the medium is acid is found as follows. The amino-acid decarboxylases have very acid pH values of optimum activity (measured in the external environment of the intact cell) which lie between 2.5 for the histidine decarboxylase of *C. welchii* and 5.5 for the ornithine decarboxylase of *C. septique* (33). Some of these enzymes have now been obtained in a cell-free condition by extraction of acetone-powders of suitable organisms (13, 36). In all the cases so far studied, the value of the pH of optimum activity for the cell-free enzyme is more alkaline than that obtained for the same enzyme in the intact washed cell; further whereas the former remains constant, the latter tends to vary from culture to culture. The degree of the difference can be seen in table 2. Doubtless other factors are involved, yet it is possible that this difference represents the difference between the internal and external pH values of the intact organism, the true pH of optimum activity being that measured with the cell-free enzyme, while that measured in the external environment of the intact cell is the external pH necessary to give rise to an internal pH equal or nearly equal to the true pH of optimum activity.

It was first noted by Cohen and Clark (9) that the rate of growth of several bacterial species is approximately uniform within a broad zone of pH in the external environment (pH 5 to 8 for *E. coli*) while, outside that zone, small pH changes have a marked effect on the rate of reproduction. This presumably means that the cell is provided with efficient means of stabilizing the internal environment within certain limits, outside of which the mechanism breaks down rapidly. It would seem that the amino-acid decarboxylases and some deaminases form an important part of this mechanism in some cells. Further we find in their formation one of the laws governing enzymic variation: the cell reacts to an adverse change in the external pH by an alteration in its enzymic consitution such that the adverse change is counteracted, and the resultant change in the internal pH is minimized. Whether this law can be generalized to cover any adverse external change is for future research to settle.

The investigations on the effect of the pH growth on enzymic constitution have brought out another interesting fact: that the formation of certain enzymes is so controlled within the cell that the activity of the cell in respect of those enzymes is constant whatever the external pH. These enzymes were classified in group 1 in the discussion above, and it was pointed out that the majority of them dealt with the removal of toxic substances. Thus, catalase decomposes toxic concentrations of hydrogen peroxide, urease decomposes urea, and alcohol dehydrogenase brings about the oxidation of alcohol. There is also a complex system of enzymes which remove formic acid: formic dehydrogenase which has an extremely high affinity for its substrate (93), and formic hydrogenlyase which is formed adaptively (90, 91) under anaerobic conditions when the dehydrogenase is presumably not effective. When formate is added to the growth medium of *E. coli* there is a decreased yield of cells, and if the medium is adjusted to a pH acid to 6.3, the organism is unable to grow at all (35), so here is definite evidence of the toxic nature of formic acid to this organism (9). We have mentioned how the formation of the formic hydrogenlyase appears to be dependent upon the need of the organism to remove formate, as the enzyme is potentially and effectively most active when growth takes place at the acid limit of formate tolerance. It would seem then that these particular enzymes play an essentially protective role by acting as detoxication mechanisms. It is difficult to understand why the distribution of these group 1 enzymes should be haphazard and not universal among the species, but it may be that these enzymes are possessed in the potential enzyme constitution of an organism only if their substrates are produced in the course of the normal metabolism of that organism. Thus, *E. coli* produces formic acid, alcohol and hydrogen peroxide in the course of its "normal" metabolism and is equipped with the corresponding detoxication mechanisms. Whether those less common strains which also possess urease are those which form urea as a normal metabolic product has yet to be discovered.

The way in which the enzymic constitution of the cell is regulated so as to minimize the effect on the cell of adverse pH change or of the addition of toxic substances to the medium at various pH levels during growth suggests that there is an equilibrium between the enzymic constitution of the cell and the constitu-

tion of the medium in which it exists, and that this equilibrium obeys Le Chatelier's Principle. This is emphasized if the theorem is stated in the form: if a system in equilibrium is subjected to a restraint, then the equilibrium shifts in such a way as to tend to annul that restraint.

E. coli can grow adequately on a medium consisting of nitrogen-free salts and amino-acids, and it is usually supposed that it does so by deamination of the amino-acids with the production and subsequent assimilation of ammonia. We know little about the processes whereby bacteria synthesize amino-acids and proteins, and the only two deaminases which are demonstrably reversible in *E. coli* are aspartase (81) and glutamic acid deaminase (1). In animal tissues, synthesis is now thought to take place through glutamic or aspartic acid followed by transamination; up to the present there would appear to be no record of transamination, other than between glutamic and oxaloacetic acids (1), occurring in *E. coli*. The glutamic acid deaminase of *E. coli*, by the nature of the variation of its formation with the pH during growth, would seem to serve the purpose of stabilizing the internal environment; and since its formation is inhibited by acid growth conditions, this deaminase would seem to be unsuitable as a means of supplying N to the cell under such conditions. When, however, we study the variation of the far more active aspartase system with pH during growth, we ·· d a very different picture (see fig. 5, ref. 35). This reversible deaminase is · ɔtentially and effectively active over the growth range of pH 5 to 8, *i.e.*, over that part of the range where the rate of reproduction is approximately independent of the external pH. It may be that this enzyme has a function different from that of the other deaminases, as it can supply N to the cell far more efficiently and over a greater range of pH than these others. *Of all the enzymes so far investigated (35, 39), this is the only one having a formation-pH curve quite of this nature.* The aspartase formation is inhibited by growth conditions alkaline to pH 8, but here the cell will acquire N from other deaminase systems whose potential activities are greatest within the pH range 8 to 10. It should be interesting from this point of view to study the variation with pH during growth of arginine dihydrolase which appears to be the only enzyme involved in amino-acid metabolism in certain streptococci (53, 32).

In addition to enzymes of the types and functions so far discussed, there are enzymes, such as glucozymase, which may be partially adaptive and which are formed to the greatest extent when the pH during growth lies in the vicinity of the pH of optimum activity. The marked increase in crop following the addition of glucose to the medium indicates that the glucozymase system releases both energy and carbon to the cell. At the same time, the presence of glucose or other fermentable carbohydrate during growth gives rise to all those alterations in the enzymic constitution of the cells which have been discussed above and which are, for the most part, without explanation at present.

When we survey in this manner the probable functions of such enzymes as have been studied by this method, it is seen that the earlier classification into constitutive and adaptive enzymes is not very informative. Thus, an enzyme may be adaptive if its function is to supply energy to the cell as in the case of

galactozymase, or if its function is to remove some toxic metabolite such as formic acid. Likewise an enzyme may be constitutive if its function is to supply an essential metabolite as in the case of aspartase, or to act as a detoxication mechanism as in the case of catalase. Therefore, detoxication mechanisms may be either constitutive, as formic dehydrogenase, or adaptive, as formic hydrogenlyase; but their presence is again restricted within the limits of the potential enzyme constitution so that, for instance, some strains of *E. coli* cannot acquire protection against urea. Further it is impossible to be certain that enzymes such as formic dehydrogenase and catalase are, strictly speaking, constitutive since their substrates are probably formed by the metabolic activities of the organism during growth. The difference between constitutive and adaptive enzymes is thus mainly of academic interest.

A great advance was made in our knowledge of bacterial metabolism when it was realized that the chemical activities of the cell must be considered as the result of the action of separate and specific enzymes within that cell; now that our knowledge concerning these enzymes and their formation is accumulating, the time has come when we can begin to understand their interdependence within a cell that exists as a result of their correlated and integrated activities.

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THE MODE OF ACTION OF SULFONAMIDES*

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CONTENTS

The following abbreviations will be used throught this review:

SA   = sulfanilamide
HOSA = p-hydroxylaminobenzenesulfonamide
SP   = sulfapyridine
ST   = sulfathiazole
SD   = sulfadiazine
PABA = p-aminobenzoic acid

A. INTRODUCTION

The field of sulfonamide chemotherapy has exhibited a mushroom growth of tremendous scope and complexity. This has resulted in considerable confusion. It is almost impossible now for an investigator to keep informed of all published works relevant to sulfonamide action. It is therefore timely that an attempt be made to gather in one place the scattered observations, both for the investigator in the field and for one desirous of learning what is known about the action of these drugs, with the object of lessening the confusion and of indicating directions for future investigation.

The primary action of sulfonamides on bacteria is generally believed to be bacteriostatic rather than bactericidal. This must be stated with certain reservations, for the action may become bactericidal if the sulfonamide concentration sufficiently high or if the presence of any sulfonamide concentration is accompanied by other unfavorable environmental conditions such as poor cultural ditions, adverse temperature, antibodies, toxic proteolytic products, etc. One of the early theories of the therapeutic action of these compounds was that the body defense mechanisms are stimulated. This idea fell into disrepute and is practically discarded (however, see section B2 for further discussion). The question of the possible inactivation of bacterial toxins is still of controversial nature and under investigation. The affinity of sulfonamides for bacterial and other proteins represents a possibility that these compounds are capable also of combining with, and thereby inactivating, toxic proteins. A final answer to this question must await the results of further studies. At present, however, the indisputable fact remains that the ultimate effect of sulfonamides is that of growth-inhibition.[1]

It is the purpose of this review to consider this growth-inhibitory action of the sulfonamides and to attempt a coordination and integration of the available information into a picture of the mode of inhibitory action of sulfonamides which the author regards as the most acceptable.

In 1940, Woods and Fildes made the discovery that p-aminobenzoic acid (PABA) is an extremely potent sulfonamide-antagonist. Therefrom arose the theory which has gained almost universal popularity, namely, that a sulfonamide interferes with the utilization of the substrate PABA in an anabolic reaction by competing with the latter for its enzyme. A careful and critical consideration of subsequent investigations reveals that this theory is based on certain assump-

[1] Most of the work leading to these views occurred early in sulfonamide research, and therefore the reader can refer to earlier reviews (160, 193) for complete discussions and references.

tions which have not as yet been proved and, furthermore, that the observations leading to the main arguments presented for its support can be equally well explained on an entirely different basis, and in fact, in certain instances *must* be interpreted on a different basis since the circumstances surrounding these instances exclude the possibility of PABA acting as a substrate. The conclusion is thus ultimately reached that the Woods-Fildes explanation for sulfonamide action cannot be the only possible one compatible with all the known facts regarding sulfonamide action and its antagonism by various substances.

As will be seen, all evidence indicates that sulfonamides achieve their bacteriostatic action by direct inhibition of one or more enzymes; this view enjoys today practically universal support. The two classes of enzymes which must be given consideration are those which catalyze anabolic or catabolic reactions and those which catalyze oxidation-reduction reactions. According to the Woods-Fildes theory the sulfonamide inhibits a hypothetical anabolic enzyme whose substrate is PABA. Others have investigated the effect of sulfonamides on oxidation-reduction enzymes, and there is considerable evidence in favor of the theory that sulfonamides inhibit cell division by a primary inhibition of one or more of these enzymes. It is this theory which is developed in the final section of this review as the one most compatible with all known facts regarding sulfonamide action. It must be forcefully emphasized at this point, however, that there is much work yet to be done before any theory of the mode of action of sulfonamide bacteriostasis can be accepted as final.

B. EMPIRICAL OBSERVATIONS ON THE ACTION OF SULFONAMIDES

The first step in the approach to the problem of a drug's mechanism of action is a consideration of the observations made on its behavior. Therefore, rather than begin with the discussion of these proposed mechanisms, it seems advisable first to acquaint the reader with certain fundamental facts relating to the behavior of the sulfonamides. This condensed account will serve to orient the reader with respect to the problem and will provide a factual background for a more critical reading of what is to follow.

1. The Inhibitory Action of Sulfonamides

The most fundamental fact about sulfonamides is that they are general cell inhibitors. A sulfonamide acts as inhibitor not only towards bacteria, but also towards other cells of practically every variety. This fact is of primary importance since it immediately casts considerable doubt that the inhibitory action of sulfonamides on bacteria is in any way unique, and that the answer to the problem of the mechanism of sulfonamide action can be sought by using bacteria alone. It is apropos, therefore, to recount briefly the various types of cells inhibited by sulfonamides, and thereby justify the use of the term "general cell inhibitor."

a. Bacteria. It is generally accepted today that the basic action of sulfonamides on bacteria *in vitro* and *in vivo* is bacteriostasis. This is not an "all or none" phenomenon; all gradations of decreased bacterial growth rate can be

produced by proper variation of the factors which influence sulfonamide action (discussed in part 3 of this section). Sulfonamides also inhibit other measurable bacterial cell functions; this will be presented in later sections. Because of its essential nature to most of the considerations in this section, it is necessary to anticipate a subject to be discussed at length in a later section (D), and state here that an exceptional feature about sulfonamide inhibition of bacteria is its complete counteraction by certain substances, foremost among which is p-amino-benzoic acid (PABA). Particular attention should be paid to references made to PABA and its action, because, according to the most popular theory, PABA is the key to the mechanism of sulfonamide action.

b. Cells other than bacteria; i. Viruses. It has been known for some time that all viruses are not alike and that there are certain groups with group characteristics. The three viruses for which there is adequate evidence indicative of a therapeutic response to sulfonamides (trachoma, lymphogranuloma venereum, and inclusion blenorrhea) and the virus of psittacosis comprise a group of viruses of large particle size which differs from typical viruses in several respects. Several attempts have been made to determine the nature of sulfonamide action on susceptible viruses. Richards et al. (220) were unable to demonstrate inclusion bodies and failed to infect baboons with pooled epithelial scrapings following sulfonamide therapy of the infection trachoma, suggesting that the drug caused disappearance of this virus. Holder et al. (98) found that contact between a sulfonamide and the virus of lymphogranuloma venereum *in vitro* results in decreased virulence of the virus but no apparent virucidal action, an observation in accordance with observations *in vivo* (112).

Poliomyelitis, choriomeningitis virus infection in mice, pneumonitis virus infection in mice, lymphogranuloma inguinale, canine distemper, and "shipyard eye" or kerato-conjunctivitis have been reported as susceptible to sulfonamide therapy, although most of these claims have been disputed. Many other virus infections which have been investigated, such as smallpox and yellow fever, have invariably been found to be unaffected.

The action of sulfonamides on the susceptible viruses is very interesting inasmuch as with applications of known methods no one has been able thus far to find evidence that viruses have a metabolism of their own. Assuming that viruses are exceedingly minute living organisms, and after making the observation that PABA counteracts the therapeutic effect of SA on mice inoculated intracerebrally with the virus of lymphogranuloma venereum, Findlay (62) extended the Woods-Fildes theory of sulfonamide action (section D1) and suggested that those viruses acted upon by sulfonamide may be those which require PABA (or a substance similar in structure) for their metabolism; for all other viruses PABA would thus not be an essential metabolite. Findlay offered as an alternative hypothesis that in the course of the metabolism of these viruses unaffected by sulfonamide so much PABA is formed by the virus that the chemotherapeutic action of sulfonamide is prevented. So far as is known today, all viruses are associated with living cells; it is possible therefore, that sulfonamide action in this instance might be indirect through an action primarily on the host cell thus rendering it an un-

suitable abode so far as the virus is concerned; however, the work by Holder et al. (98) already referred to, indicates that, in the case of lymphogranuloma virus, at least part of the action is directly on the virus itself.

On the other hand, if the virus is a non-cellular entity, it is possible that the action may be direct by interference with its autocatalytic and self-propagative properties. This interference may be in the nature of an adsorption of an inhibitor on the virus itself.

ii. Protozoa. Malaria (Plasmodia):[2] Reports of the effectiveness of sulfonamides on human malaria have been somewhat conflicting. There have been many uncertain or unfavorable and many favorable reports with regard to the therapeutic effectiveness of sulfonamides against human tertian malaria (*Plasmodium vivax*), quartan malaria (*P. malariae*), and estivo-autumnal malaria (*P. falciparum*).

Repeatedly, sulfonamides have been found to be effective against the virulent *P. knowlesi* infection in rhesus monkeys, while they exert no effect on the milder *P. cynomolgi* and *P. inui* infections (33, 34, 35). It appears in general that the sulfonamides are more effective against the more virulent plasmodia.

Certain avian forms of malaria such as *P. praecox* (3) and *P. circumflexum* (186) have been reported as affected by sulfonamides, but *P. lophurae, P. cathemerium, P. nucleophilum, P. relictum*, and *Hemoproteus columbae*, on the other hand, have all been claimed by various workers to be resistant to sulfonamide therapy.[3] In the case of *P. lophurae* infection in ducks, however, there have been several favorable reports, and Marshall et al. (188) demonstrated the importance of the blood concentration-time curves in the sulfonamide therapy of this particular infection, showing that maximum effect can be obtained only by keeping the blood sulfonamide level up for a sufficient length of time. It was the opinion of these investigators that the differences in response to sulfonamides of monkey and human malaria on the one hand and avian malaria on the other is at least partly due to differences in the blood concentration-time curves, especially when single oral doses are administered daily. Some of the discrepancies observed in the sulfonamide therapy of different malarial infections undoubtedly are due to species differences in susceptibility to the sulfonamides (34, 188).

PABA antagonizes sulfonamide action on *P. gallinaceum* (182) and on *P. lophurae* (188, 240), but does not antagonize the action of quinine and atabrine, thus indicating that these drugs act on plasmodia through a different mechanism than that of sulfonamides.

Other protozoa: Amebae, paramecia, trichomonads, (80), *Toxoplasma* (229), *Leishmania tropica* (242), and *Entamoeba histolytica* (222) have been reported as inhibited by sulfonamides. Cell division of the flagellate, *Polytomella caeca*, is blocked by SA, this inhibition being antagonized by PABA (163, 164). The flagellate which is inhibited has a volume four times that of the normal average. This is in agreement with numerous analogous observations on bacteria and

[2] For references of the effectiveness of the sulfonamides in malarial infections see the review by Williams (280).

[3] See Marshall (187) for a review of the effect of sulfonamides on avian malarias.

leads to the interesting hypothesis that sulfonamides, within certain limits, inhibit the division of microörganisms rather than growth primarily (164). In the work on *Hydra* (referred to below), however, a similar observation was made but was ascribed to body edema, perhaps brought about by a change in membrane permeability. Both are distinct possibilities but at present the exact cause and nature of this cell volume change is not known.

iii. Other cells. *Halteria*, *Hydra*, *Mesostoma* (flatworm), *Stenostomum* (rotifer), *Dero* (annelid) (59, 148, 149), chick embryo heart tissue culture, bone marrow, wound healing, various yeasts (*Torulospora*, *Torula*, and *Saccharomyces*), various fungi (*Trichophyton gypseum*, *T. purpureum*, *Blastomyces dermatitidis*, *Aspergillus niger*, and *Neurospora crassa*), *Actinomyces hominis* and *A. bovis* (also the clinical infection, actinomycosis), and higher plants (algae, a diatom, *Tradescantia occidentalis*, *Lupinus albus* roots, tomato roots, and *Pisum* roots) have all been reported as inhibited by sulfonamides; the concentrations of sulfonamides required to produce such an effect vary considerably, anywhere from a few mg % to over 100 mg %. PABA counteracts the sulfonamide inhibition of yeast (139) of *T. purpureum* and *T. gypseum* (43, 44), of *Aspergillus niger* (164), of *Neurospora crassa* (268), of algae (30) of a fresh-water diatom (278), of tomato roots (16), and of rootlets of *Pisum* and *Lupinus albus* (183).

Sulfonamide action on plants is of a somewhat unexpected nature. Thus, mitotic irregularities, chromosomal rearrangement, polyploidy resulting in large cells and strange new plant varieties some of which are giants, and transformation into degenerate variants have been observed. Certain of these effects are reminiscent of the action of colchicine. The improbability of any such hereditary mechanism playing a part in the dissociative changes occasionally observed in bacteria under the influence of sulfonamides has been discussed at length by Mellon (192).

Liver tissue, sea urchin eggs, bacterial luminescence, and luminescence of *Cypridina* are also affected by sulfonamides and will be considered in detail in section E8.

Next to the fact that sulfonamides inhibit all these various cell types, the most interesting observation is that in most instances PABA can completely counteract this inhibition. This fact can only strengthen the doubt that the inhibitory action of sulfonamides on bacteria is in any way unique. The tentative conclusion can, therefore, be made that the mechanism of sulfonamide inhibition is fundamentally similar, if not identical, in all cells susceptible to sulfonamides. This certainly would not be unexpected, for it must be remembered that in its gross details the metabolism of bacteria is very similar to that of most other types of cells.

2. The Biphasic Action of Sulfonamides

It is a rather general phenomenon that substances toxic to cells will also stimulate the cells at sub-toxic concentrations, e.g., it is seen with nicotine, narcotics, cyanide, actinomycin, and many others. Thus it is not surprising that low concentrations of sulfonamides stimulate bacterial growth (63, 83, 135, 136).

The primary stimulation of growth by the sulfonamides (before growth-inhibition occurs) observed by many investigators (63, 82) may well be an expression of the same phenomenon. The stimulatory action of sulfonamides is by no means confined to bacteria, for it has been demonstrated in the production of polymorphonuclear leucocytes by bone marrow (63), in phagocytic activity (27), in plant and yeast growth (81, 135, 166), in amplitude of dog heart beat (195) and in the multiplication of *Entamoeba histolytica* (5).

Following the demonstration of the growth-promoting activity of SA in plant and yeast growth, comparable even to indolyl-3-acetic and 1-naphthyl-acetic acids, Grace (81) pointed out that it is difficult to know where to draw the line between substances commonly regarded as inhibitors and those regarded as stimulants. For example, the phytohormone indolyl-3-acetic acid in high concentrations inhibits cell growth in a manner similar to the sulfonamides, and high concentrations of the sulfonamide-antagonist PABA will inhibit bacterial growth very effectively (cf. DId). According to Grace, low concentrations of the sulfonamides may actually stimulate an infection, their therapeutic effect in such bacterial infections being due to a sufficiently high concentration. This, of course, has a serious clinical implication, namely, the possible danger of underdosage. In certain instances the overall therapeutic effect of these compounds, at ordinary dosage levels, may be a combination of two factors: first, an inhibitory action on the bacteria which are very susceptible to the sulfonamide, the sulfonamide concentration exceeding the range in which stimulation of the bacteria occurs; second, a stimulation of the host's tissues (bone marrow, phagocyte activity, general tissue resistance) which are less susceptible to the sulfonamide whose concentration here lies within the range in which tissue stimulation does occur (the concentrations required to inhibit such tissues *in vitro* have been found usually to be much higher than the levels attained therapeutically *in vivo*). Such a concept receives some support in the preliminary reports of Mellon *et al.* (194) where it is stated that ST, but not SA, increases the oxygen consumption of certain tissues as measured *in vitro* by the Warburg technique. The observed stimulation, however, may have been due to oxidative autolysis of the tissues. In any event, this phenomenon of stimulation in low concentrations and inhibition in high concentrations is not yet understood.

3. Factors Influencing Sulfonamide Action

The intensity of the action of all drugs depends to a great extent on environmental conditions. This is true of cell inhibitors, and sulfonamides are no exception. Much research has been expended upon the effect on sulfonamide activity of certain environmental conditions which are easily varied. Several of the observations made could have been predicted, while one, the effect of pH, has been only recently explained with any degree of satisfaction, and another, the effect that the size of bacterial inoculum has on sulfonamide activity, still defies comprehension. It is important to keep in mind, however, that these studies and their conclusions fit equally well the various hypotheses on how sulfonamides achieve their inhibitory action.

a. Drug concentration; the adsorptive nature of sulfonamide action. Bacterial growth-inhibition by sulfonamides appears to obey the law of mass action, which requires first that the inhibition be reversible, and second, that the inhibition be directly related to inhibitor concentration. This relationship is seen with most cell inhibitors. With respect to sulfonamide inhibition, the first requisite is satisfied by two independent and well-established observations, namely, the inhibition can be reversed by removal of the sulfonamide (157, 169), and the inhibition can be antagonized by PABA and other sulfonamide-antagonists (cf. Section D). PABA-antagonism of sulfonamide inhibition has been shown to obey the law of mass action (cf. D2b) which could not be unless the inhibition itself obeyed the law.[4] The second requisite, that the inhibition be directly related to sulfonamide concentration, has been observed by numerous investigators (27, 82, 126, 154, 193); table 1 gives typical data demonstrating this point. As indicated in some reports (126, 205), this proportionality may not, in every case,

TABLE 1

Showing the degree of bacteriostasis as observed in blood-agar plates containing different amounts of SA and inoculated with a virulent culture of Streptococcus pyogenes

Data from Table IX of Mellon et al. (193)

SA CONCENTRATION		NUMBER OF COLONIES	INHIBITION
M	mg %		
			%
0 (control)		206	
0.00006	1	107	48
0.0006	10	92	55
0.006	100	91	56
0.0075	125	84	60
0.01	167	79	62
0.015	250	59	71
0.03	500	52	75

be strictly linear over a large range of concentrations. If, as assumed by some (Section D), sulfonamides affect more *loci* in the cell as their concentration is increased, one would expect to observe occasionally such discontinuities in relationship.

A rather interesting question is whether sulfonamide action takes place at the cell surface or within the cell. There is the definite possibility that bacterial metabolism in general takes place predominantly at the surface (2);[5] if this were

[4] In view of the competition which exists between sulfonamides and PABA it may almost be assumed that PABA action is also a result of reversible combination. Other sulfonamide-antagonists (Section D) apparently do not act competitively, but this by no means precludes their combining by a reversible combination.

[5] The term "cell surface" as related to bacteria is ambiguous since it has been demonstrated by staining techniques that surrounding the inner protoplasm of the cell there is the protoplasmic membrane, outside of which is the cell-wall (125). Studies with the electron microscope (203, 204) have confirmed the existence of a solid cell-wall distinct from

true, it would almost be assumed that sulfonamide action occurs at the surface, since there is little doubt that this action is primarily one of inhibition of some metabolic function.   Feinstone *et al.* (56, 57) observed that strong adsorption or diffusion through the cell is not a criterion of drug activity, and claimed to have obtained evidence with the aid of the electron microscope that the drugs do enter the cell.   This, of course, would not rule out the possibility that the inhibition produced is due to enzyme inhibition at the cell surface; it would depend on the location of the functional enzymes which are interfered with.   Electrokinetic experiments have revealed that SA and PABA behave alike at the bacterial surface, thus suggesting that their action is at the surface (18).

The combination of sulfonamide with whatever component of the cell it inhibits may be regarded as an adsorption.   The term "adsorption" is variously understood today.   As used throughout this review it is meant to imply an interaction at a surface, for instance at the surface of an enzyme molecule.   Such interaction between two molecules may be mediated through electronic van der Waals attraction, attraction of electric dipoles or multipoles, Coulomb attraction,

TABLE 2

*Effect of varying the number of organisms inoculated on the per cent of the control population;
data from Table 2 of Libby (155)*

ORGANISM AND DRUG TESTED	% OF CONTROL POPULATION WITH AN INOCULUM PER 10 ML OF:		
	50 million	25 million	12.5 million
Type II pneumococcus:			
Sulfapyridine	61	51	44
N⁴-sulfanilyl sulfanilamide	54	44	38
Paratyphoid A:			
Sulfapyridine	77	57	46
Streptococcus C493:			
Sulfanilamide	6S	57	34

hydrogen bond formation, or primary chemical valence.   It is impossible at present to say which form of interaction applies to sulfonamide action, but as long as the interaction is reversible the law of mass action must apply equally well to each.   This allows one in theoretical considerations to treat them as one and the same.

b. Size of inoculum.   In the presence of a constant amount of sulfonamide, the inhibition of bacterial growth is inversely related to the number of organisms present (20, 27, 126, 154, 162, 177, 260); or, in other words, as the size of inoculum is increased a greater amount of sulfonamide is required to produce the same inhibition (see table 2 for a typical set of data).   Apparently in some cases inoculum size has little effect (17, 181), but these have certainly been in the

the inner fluid or potentially fluid protoplasm.   It is impossible at present to say at which locus, protoplast, the protoplasmic membrane, or the cell-wall, metabolic reactions would be most likely to occur.

minority. Nitti *et al.* (211) claimed that the importance of inoculum size varies with the organism, being of less importance with meningococci and gonococci than with streptococci and pneumococci, and of no significance whatsoever in growth-inhibition of the mold, *Aspergillus niger*.

If the ratio of volume of medium to volume of bacteria were not so high in such experiments one would suspect that, where this inverse relationship is observed, reduction of concentration resulting from adsorption of the sulfonamide from the medium by the bacteria was a factor. Several checks on this possibility have revealed either no measurable, or only a very minute decrease in sulfonamide concentration after contact with bacteria (126, 177). It would appear, therefore, that for the production of a certain level of inhibition of bacterial growth, there is, in most instances, a trend in the direction of a definite number of molecules of sulfonamide being present per bacterium. This direct relationship is not seen between cell suspensions and various other inhibitors such as narcotics, nor would it be expected. The relationship of amount of an enzyme per cell to the inhibitor concentration is the determining factor in such a heterogeneous system. This relationship does not change by varying the number of organisms in a given system. The observed inverse relationship between sulfonamide effect and inoculum size must therefore be due to some unknown variable.

Since certain bacteria have been shown to produce sulfonamide-antagonists, which in the case of some organisms can be found in the medium, it would at first seem quite logical that this should be the answer to the problem. The larger the inoculum the greater the amount of antagonists produced, and thus the greater the amount of sulfonamide required. This very promising explanation was apparently shattered when no antagonists were found in the medium of *Escherichia coli* and of pneumococcus cultures, although in both instances the inverse relationship between sulfonamide concentration and inoculum size existed (126, 162). Furthermore, if production of sulfonamide-antagonist were responsible for this phenomenon, and if the antagonist produced by bacteria is PABA, as believed by some, the inverse relationship should not be observed with HOSA as inhibitor, since PABA does not reverse HOSA action (section *C*); however, the inverse relationship does exist (20). Here is an empirical observation which appears to be inconsistent with general chemical principles and at present defies explanation. The paradox awaits clarification.

c. Composition of the medium; in vitro vs. in vivo. Occasionally throughout this review the sulfonamide concentrations[6] used in various investigations will be cited, presumably to provide some basis for comparison of results. Actually, however, such comparisons in most instances are of little significance. The reasons for this statement will become apparent after reading the section on sulfonamide-antagonists (D). Suffice it to say at this point that the extreme variability in the amounts of sulfonamide-antagonists in various media alone makes comparison of results *in vitro* practically impossible (154, 179, 180, 262,

[6] Throughout this review, for the convenience of the reader, all concentrations will be expressed two ways, in molarity (M) and in milligrams per cent (mg %).

263, 265). With the little that is known of the effects of various components of culture media on bacterial metabolism, and in view of the extreme variability of media employed in sulfonamide research, it seems utterly hopeless to attempt to evaluate the results of every investigator in the light of the medium employed.

A good example of the role played by the medium is the observation by Mac-Leod and Mirick (180, 181) that properly prepared fresh calf-liver infusion has no sulfonamide-counteracting action, and to produce the same results in plain broth and peptone broth, 20 and 40 times, respectively, as much sulfonamide is required. Another fact which complicates the situation is that if the medium is not optimal for growth, sulfonamide action is more pronounced (179, 259). Dilution of cultures with water, physiological saline solution, or buffer solutions either kills or injures some cells (181). This probably explains the observation that increasing the sodium chloride concentration of the medium results in increased sulfonamide action (160, 193). This is presumably another example of supplementation of adverse influences on the bacterial cell.

Since sulfonamide-antagonists have been found in every body tissue so far examined, it would be expected that there would be some lack of correlation between sulfonamide activity *in vitro* and *in vivo*. White et al. (277) found that, although no sulfonamide is active *in vivo* unless it is active *in vitro* or can be decomposed to a compound which would be active *in vitro*, compounds can be active *in vitro* while inactive *in vivo*. This lack of complete correlation between activity *in vivo* and *in vitro* has been observed by others.

d. pH. Varying the environmental pH has a very definite effect on sulfonamide activity. This was first noted by those interested in sulfonamide therapy of urinary tract infections, an instance where the pH can be controlled within certain limits. Investigation showed that sulfonamide activity in urine is increased as the pH is raised, e.g., from the range 5.5–6 to 7.5–7.8 (93). These investigations of sulfonamide activity at various urine acidities were made primarily in quest of an answer to an important clinical question, and not to ascertain the nature of the effect of pH on sulfonamide activity. Subsequent work designed specifically to give such information seemed at first to indicate two things: first, that it is the anion which is the active agent in a sulfonamide solution (which is of course compatible with the earlier observations that sulfonamide activity increases with urine pH) (75, 235), and second, that ion for ion all the sulfonamides are approximately equal in activity (75). Thus, the activity of a sulfonamide at any pH would be governed only by its acidic dissociation at that pH.

Cowles (37) and Brueckner (24), after making careful comparisons of bacteriostatic sulfonamide concentrations with ionization curves, found that an amendment would have to be made to the simple "ionic" theory. They observed that, in general, sulfonamide activity is at a maximum when the pKa of a sulfonamide is close to the pH of the culture medium, and decreases progressively as the pKa values depart in either direction from this pH. The maximum activity is, therefore, at the pH where 50 per cent is in the ionized form and 50 per cent in the non-ionized form. This suggests that only the non-ionized form can enter the

cell and, once in, only the ionized form is active; several examples are known where the intact organic molecule is better able to penetrate the cell membrane than the ions. As the pH departs in either direction from the pKa value, sulfonamide activity decreases. As Brueckner recognized, the mathematical approach he and Cowles made to the problem of sulfonamide activity is empirical.

Up to this point, all attempts at explaining the relative activities of sulfonamides were based on correlating in some way the activities with the acid dissociations of the compounds. There is no disagreement that pH does affect sulfonamide activity; but the "ionic" hypothesis met certain difficulties. Further analysis of the data used in its support revealed that the "ionic" interpretation was unjustified (128). Furthermore, such facts as the activity of compounds incapable of ionization, e.g., SG, the increase in activity of undissociable sulfonamide molecules with an increase in pH, and the decrease in activity of sulfonamide anions with an increase in pH (232) cannot be explained on such a basis. This would indicate that possibly the pH and the acid dissociation of the compound are not the only factors involved, and in fact may be only secondary to some more basic variable.

Bell and Roblin (12), aware of the inadequacy of the simple "ionic" theories, and seeking an approach to the problem which would utilize some fundamental physical property related both to structure and activity, observed that the more negative the SO_2 group of a sulfonamide, the greater the activity. They pointed out that the more negative the SO_2 group, the more closely it resembles the COO^- group of PABA at pH 7, for at this pH the carboxyl group of PABA is over 99 per cent ionized and consequently carries a negative charge. Since the SO_2 group of a sulfonamide in the ionized form is much more negative than in the non-ionized form, the former should be much more active than the latter. This theory therefore allows for an activity of undissociated sulfonamides. They disagreed with the contention (75) that ions of different sulfonamides are equally active, since the experimental data indicate that the ions of stronger acids are less potent. Since the electron-attracting power of the R group (on the N attached to the SO_2 group) is proportional to the acid strength, it follows that the greater the acidity, the less negative the SO_2 group of the ionic and molecular forms, and the less the activity of both. Up to a certain point this decrease in activity with increasing acid strength is more than compensated for by an increasing proportion of the active ions. Beyond this point, an increase in acidity is not paralleled by a proportionate increase in ions; therefore, the dominating effect is now the decreasing negative character of the SO_2 group which is accompanied by a decreasing activity. The activity of the undissociated forms should show a continuous increase as acid strength decreases. Thus, when the compounds become such weak acids that the effect of the highly active ions is negligible, the curve relating pKa to activity should pass through a minimum and then increase as acid strength decreases. On the basis of these theoretical and experimental considerations, Bell and Roblin believed that the optimum of N¹-substituted SA derivatives has been reached, insofar as inherent bacteriostatic activity is concerned.

Kumler and Daniels (134) have recently presented theoretical considerations indicating that a fundamental factor for activity is the contribution of the resonating form of the compound with a coplanar amino group. The negative character of the SO_2 group is thus a concomitant factor associated with the resonating form. Observations at variance with pre-existing theories are reconciled by this new theory. Compounds which appeared to be exceptions to the Bell-Roblin theory (sulfanilylurea, sulfanilylguanidine, sulfanilamide-1,2,4-triazole), and those which do not fall within the scope of their theory (sulfones, ring N-methyl and N-methylsulfapyridine and sulfathiazole compounds), can be adequately accounted for on the basis of resonance. Furthermore, it is seen that, so far as activity is concerned, whether the most active species of the compound is the anion, cation, or neutral molecule is an incidental property. Kumler and Daniels agree with Cowles and Brueckner in their explanation for the maximum in the activity vs. pKa curve for N^1-mono-substituted sulfanilamides, that the undissociated molecules get to the site of action more easily but that once there it is the ion that is more active. They point out, however, that the optimum ratio of ions to undissociated molecules need not be 50/50, but could be almost any ratio, depending on the relative rates of the two separate reactions.

It might be expected that pH would also influence the sulfonamide-antagonistic activity of PABA. If either the undissociated or the ionic form of PABA is more active than the other, then the pH should affect PABA action. Lwoff et al. (164) reported that the antagonistic activity of PABA parallels its dissociation curve, being maximum at its isoelectric point, in the case of SA-inhibition of the flagellate Polytomella. It was considered that the undissociated form of PABA penetrates more readily into the cell than the ion. These authors claimed that PABA-activity varies little or not all with pH with respect to E. coli or Aspergillus niger. If this were true, it might be interpreted to mean that, in these latter two instances, it is not necessary for the PABA to gain entrance to the cell in order to exert its action.

Brueckner (24), however, found that when pH was varied in cultures of Staphylococcus aureus, changes occurred in the molar ratio, sulfonamide/PABA, which did not correlate with changes in drug activity. This indicated that PABA activity might also be changing with pH variation. Experiments demonstrated that PABA does not increase in activity as its ionic concentration increases; it became decreasingly effective as the pH rose from 6 to 9, the range in which PABA rapidly becomes more than half dissociated. Thus, it is seen that the molar ratio of sulfonamide/PABA at varying pH levels cannot be interpreted exclusively in terms of sulfonamide activity. If the sulfonamide loses potency with increasing pH at the same rate as PABA, then no change in molar ratio should occur as the pH is increased. This was found to be the case with SD and ST at pH levels of 7 and above. If, on the other hand, the sulfonamide becomes increasingly active as the pH rises, as SA does, then changes in the molar ratio should occur; this was observed experimentally.

Fisher et al. (68) found that apparently only the non-ionized form of PABA is active as a growth inhibitor in fertilized sea urchin eggs. This may mean that, here again, only the non-ionized form can penetrate the cell, although once

in it may be the ion which is active (in this instance as an inhibitor rather than as an antagonist). This appears to be true in fertilized sea urchin eggs for the inhibitory actions of local anesthetic bases which have PABA or benzoic acid as their base (132). The effect of pH on the fungistatic activity of PABA and benzoic acid, and on the inhibitory action of benzoic acid on yeast fermentation and bacterial growth, also point to such a conclusion (39, 97).

In any event, it seems that this problem of pH-effect on PABA activity and that of other sulfonamide-antagonists requires clarification. Inasmuch as most culture media contain sulfonamide-antagonists and the organisms themselves seem capable of producing them (cf. D2c), the evaluation of investigations on the relationship between pH and sulfonamide activity cannot be on a sound basis without further knowledge. The problem is by no means simple; changes in pH might also affect membrane permeability, the enzymes, etc.; then, too, there is the effect of proteins, salts, etc. on the ionization and neutralization of the sulfonamide, of PABA, etc.

e. Temperature. The bacteriostatic and bactericidal action *in vitro* of the sulfonamides is definitely increased by an increase in temperature (7, 150, 151, 276). Several investigators have compared the action at approximately 37°C (human body temperature) and at several degrees higher. It was found that the increase in temperature *per se* is very definitely not the sole cause of the observed effects. This, of course, is of clinical interest, as will be seen below. In some instances, a particular concentration which is bacteriostatic at 37°C becomes bactericidal at slightly higher temperatures. The most quantitative experiments were those on streptococci reported by White (1939) who found that at 30°C SA concentrations less than 0.058 M (1000 mg %) are inactive, at 36°C concentrations less than 0.0058 M (100 mg %) are inactive, and at 39°C concentrations of 0.00058 M (10 mg %) or less are bactericidal. About 100 times as much sulfonamide was required at 37°C as at 39°C to produce the same effect. As the temperature is increased above 37°C, the ability of PABA to antagonize sulfonamide action decreases markedly (150, 151).

That increased temperature results in increased sulfonamide activity is also apparent *in vivo*. This has been observed in gonococcal infection of the chick's chorioallantoic membrane (8), in pneumococcal septicemia of rabbits (193), in human gonococcal infection where artificial fever is employed in conjunction with drug therapy (7, 8), and in infected wounds (85).

The facts indicate (section B3b) that the reaction in which the sulfonamides are involved is of an adsorptive nature. Most adsorptions are strongly exothermic, and it may be said that as a general principle such an absorption decreases with increasing temperature. Many adsorptions, however, apparently are not so simple because they do not follow this rule; e.g., they may increase with temperature up to a certain point and then decline. "Activation" energy and other factors are thought to be the cause of such phenomena, but as yet they are by no means completely understood (2). Studies of the effect of temperature on inhibitions (of an adsorptive nature) of enzyme systems are even more difficult to interpret since, besides the various factors involved in the ad-

sorption process *per se,* the effect of temperature on enzyme activity and probably other factors are to be considered. An example of the effect of temperature on enzyme inhibition is seen in the work on sulfonamide inhibition of bacterial luminescence (106, 107). Depending on the circumstances, a rise in temperature can increase or decrease the inhibition.

f. Age of culture; length of experiments. Though it may be that, qualitatively, the action of sulfonamides on bacteria is the same regardless of the age of the culture, it would be expected that the action would vary quantitatively with age. Critical data on this particular problem are very scanty and conflicting. Long and Bliss (160) claimed that the effect of SA is less on young cultures than on old ones. On the other hand, MacLeod and Mirick (18) reported that, as is seen with various unfavorable agents such as bacteriophage, water, etc., older cells are more resistant to sulfonamides. The ages of the cultures used by various investigators have differed considerably, and it seems probable that in many of the quantitative differences observed, this factor has played an important part. The previous history of the bacteria, i.e., their previous environment and growth conditions, may also be important.

The lengths of the time allowed to elapse before experimental observations are made also have varied tremendously, extending from fractions of an hour up to almost a week. It is a hopeless task to attempt to keep an actively growing bacterial culture constant in every respect over any but the shortest period of time. Though the concentration of sulfonamide does not change, other factors which influence its action change considerably with time: the pH changes, sulfonamide-antagonists are produced by the bacteria, bacterial substrates are used up, poisonous products accumulate, the bacteria age. Even if the bacterial growth is completely inhibited a certain amount of metabolism persists, and there is the possibility that the culture may "escape." This "escape" may be brought about by either a sufficient number of cells autolyzing and releasing substances which reverse the sulfonamide effect (103) or an acquisition of drug-fastness (103, 237). In instances where bacterial growth is only retarded, the bacteria may within a relatively short time resume a growth rate equal to the control. This may be partially due to development of resistance; but this would be unnecessary for, as soon as the number of bacteria reached a certain range, the amount of sulfonamide present would no longer be inhibitory (cf. B3b). It can be calculated from the data of Sevag and Shelburne (247) that sulfonamide effect may change from hour to hour even in the early hours of an experiment.

It is, therefore quite possible that, by varying the lengths of experiments, qualitative as well as quantitative differences in results may be obtained. Such results would lead to logical but spurious conclusions. A good example of this has been emphasized by Lewis and Snyder (154) who were faced with the paradox of organisms growing better with SA than without it. It was discovered, however, that the results were due to a continued reproduction near a maximum over a longer period than in plain broth. The SA was actually depressing.

g. Sulfonamide structure. It is not within the scope of this review to consider in any great detail the tremendous mass of literature dealing with the effect of

chemical structure on sulfonamide activity (212). The theoretical approaches to this problem supplied by Bell and Roblin and by Kumler and Daniels have already been presented. A few of the more important empirical principles, however, may be mentioned.

After it was firmly established that the activity *in vivo* of Prontosil and similar compounds is due to their breakdown to SA, investigations were planned to determine whether the *p*-aminobenzene-sulfonamide nucleus is the essential basis for all sulfonamide activity.

That the *p*-amino group is not necessary for activity was illustrated by various findings. Compounds in which the *p*-amino group is oxidized show activity, in many instances considerably greater than that of SA. Nitrogen itself does not appear to be required in all cases for activity, e.g., 4,4'-diacetyldioxyphenyl-sulfone is an effective agent (152). With few exceptions, however, all active compounds contain a nitro or amino group in *para*-position to the sulfur. Kumler and Daniels (134) are of the opinion that the evidence indicates the amino group to be the functional part of the molecule for activity. The generally accepted fact that no sulfonamides with a substituted *p*-amino group are active, and the general chemical inactivity of the SO_2 group as compared with the very reactive amino group support this conclusion.

Nor is the SO_2 group essential as is evidenced by the activity both *in vivo* and *in vitro* of *p*-nitrobenzoic acid (120), and the activities *in vitro* of *p*-amino-thiophenol and 4,4'-diaminodibenzene disulfide, both of which are antagonized by PABA (84). It has been suggested that the SO_2 part of the molecule may be responsible for certain of the toxic reactions caused by sulfonamides (224, 281). The nature of the sulfonic end of the benzene ring may determine the diffusibility into or affinity for bacterial cells (252); its effect may also be indirect, through its influence (including that of R substituents) on the *p*-amino group (84, 134).

Inhibition of carbonic anhydrase, on the other hand, depends on an unsubstituted SO_2 group but not a *p*-amino group (184).

A rather interesting series of investigations has revealed the fact that the sulfur of active compounds can be replaced by other elements (arsenic, carbon, phosphorus, selenium, tellurium) and retain activity which, in some cases at least, is even increased (84, 225). Collier (36) is of the opinion that, in such cases where substitution of the sulfur results in increased activity, the polarity of the molecule has been increased, thus intensifying the reactivity of the active group (presumably the *p*-amino group).

Since there is no longer any doubt about the fact that PABA is able to reverse true SA-like activity both *in vivo* and *in vitro*, we now have a qualitative biological test (the specificity of which may be questioned, however), whereby it can be determined whether or not a substance exerts its activity by the same mechanism as SA, SP, SD, etc. It is quite possible that many of the compounds referred to above which are unlike SA in basic structure, although possessing definite anti-bacterial activity, act by a mechanism dissimilar from that of SA. Accordingly, a reëvaluation of the activities of all these various compounds must be made. Thus, Green and Bielschowsky (84) found that with but one

exception, replacing the sulfur by selenium or tellurium gives very active compounds which, however, are not antagonized by PABA. From this it would appear that the $NH_2\langle\ \rangle S$ nucleus may be basic for true SA-like activity after all (84). Kuhn et al. (133), however, observed PABA antagonism of 4,4'-diamino-benzophenone, 4,4'-diamino-diphenylsulfone, phosphanilic acid and carbopyridin; this indicates that, using PABA reversal as a criterion, the sulfur is not indispensable for true SA-like action.

This particular problem is not only important from the standpoint of furthering the understanding of the mode of action of SA and related compounds, but also because it opens up the definite possibility of new therapeutic anti-bacterial agents which are more active, not being counteracted by sulfonamide-antagonists. Presence of the latter is frequently the cause of therapeutic inefficiency with SA, SD, ST, etc.

4. Delay vs. no Delay in Sulfonamide Action

A point of some importance has been the almost universal observation that sulfonamide action in vitro and in vivo is delayed approximately from 2 to 6 hours after primary contact between sulfonamide and organism (17, 157, 162, 177, 205). Probably its greatest importance lies in the interpretations based on it. Thus, those who championed the theory that SA has to be transformed into an active substance before any effect occurs, claimed that the delay in action corresponds to the time of sulfonamide activation. In fact, it was the belief of some that the oxidative processes of bacterial multiplication were required for the conversion. As will be seen in section C, it seems fairly certain now that this idea must be abandoned. Then many of those who believe that sulfonamides interfere with bacterial nutrition have expressed the opinion that the slow development of bacteriostasis parallels the gradual depletion of bacterial stores of essential metabolites whose formation has been inhibited by sulfonamide action. It must be noted that such an interpretation presupposes that such action is an inhibition of the formation rather than the utilization of the essential substance, the latter being the foundation of the original proposal of Woods and Fildes (cf. section D1c).

A strong argument against the latter thesis is that in certain instances such a great delay in action is not seen (126, 163, 247), e.g., inhibition has been observed as early as 15 minutes following contact with sulfonamide.

Those instances in which the delay is relatively short (say of the order of 10 to 40 minutes) can be readily explained on the basis of the adsorptive nature of sulfonamide inhibition (section B3a). Adsorptive processes, unlike interactions between most molecularly dispersed reactants, are often slow to reach equilibrium. This has been tendered as a possible reason for the delay of sulfonamide action when observed (27, 205). Cowles (37) reported that he was able to show that it takes time for sulfonamide to enter bacteria and for equilibrium to be reached. Since distribution of the drugs varied, it was suggested that the rate of diffusion of the sulfonamides may be a contributory factor. Of course, as already stated, it is not known whether actual penetration of the cell

bacteria (75, 85, 287), this non-specificity extending even to the tubercle bacillus (70). Green and Parkin (85) also claimed that the sulfonamides are not specific for different organisms, as judged from the local action in infected wounds as well as from studies *in vitro*. The first careful approach to this problem *in vivo* (not wound infections) was made by Marshall *et al.* (189). Thirty-three typical SA derivatives were compared on streptococcal infection in mice, the comparative therapeutic effect being based on blood concentrations giving the same response. On a weight (mg %) basis, no derivative was significantly more active than SA; on a molar basis, SP was more active. On the other hand, all derivatives but one were more active than SA against pneumococcal infection. SP, ST and SD were also much more active against infection by *E. coli* than SA. It was logically concluded therefore that there is a definite specificity of sulfonamides on bacterial infections in mice.

There seems, therefore, to be a great discrepancy between results obtained *in vitro* and those obtained *in vivo*. The data published by Marshall *et al.* (189) reveal that some sulfonamides worked better *in vitro* than *in vivo*, and *vice versa* (no *in vitro* data were published on SP, SD, or ST, however). A rather attractive explanation is that the various sulfonamides are not specific for certain bacteria as borne out by the *in vitro* studies, but that certain sulfonamide-antagonists present *in vivo* are capable of greater antagonism towards the action of one particular sulfonamide on one bacterium than on another. In support of such a hypothesis is the finding that serum *in vitro* antagonizes the inhibitory action of SA and ST on pneumococci and staphylococci but not on streptococci (246). This of course would explain why SA *in vivo* may be specific for streptococcal infection. Because of its great clinical importance, this problem of sulfonamide specificity deserves more thorough investigation.

b. Different drugs on one bacterium. It is beyond the scope of this review to consider in detail the relative activities of the many sulfonamides which have been prepared, and the effects which various substituents, side chains, etc., have on activity. Some phases of this problem have already been touched on. It may be stated, however, that the main question at present is whether the change in activity associated with a change in sulfonamide structure is due to a change in basic mechanism of inhibition, or merely due to an enhancement or suppression of the same mechanism brought about by an altered adsorption coefficient or acid dissociation constant. Various theories attempting a correlation of sulfonamide activity with certain physical characteristics of the molecule have already been discussed (B3d). Further evidence, *pro* and *con*, for each of these two possibilities will receive some consideration in subsequent sections; it seems safest to conclude in favor of neither at the moment.

6. "Sulfonamide-fastness"

a. Natural resistance. One of the least understood of the phenomena of sulfonamide action is the extreme variability in susceptibility of various bacteria to the action of the compounds *in vitro* as well as *in vivo*. Even various strains of one species vary greatly (145). It has been shown, however, that all bacteria are more or less susceptible to sulfonamides (82, 84).

Attempts to explain this variance have been made on the basis of the anticatalase theory

of sulfonamide action and the Woods-Fildes theory; these will be discussed later and will be found to be inadequate. Other possibilities which must be considered include differences in the ability of the sulfonamide to penetrate the cell (if this is necessary) and differences in enzyme patterns. For example, Felsenfeld (58) studied various mannitol-fermenting strains of *Shigella sonnei* and found that colony forms with the greater fermentative activity were the more resistant to sulfonamides as well as to adverse physical conditions such as heat and drying.

Part of the general success of sulfonamide-therapy in the treatment of a particular infection probably depends on the capacity of the organism involved to stimulate antibody formation, although differences in antigenicity of strains are not primarily responsible for the variation in effectiveness of sulfonamide therapy (236).

b. Acquired resistance. Quite a large volume of literature has appeared reporting the acquisition by bacteria of increased resistance to the sulfonamides, both *in vitro* (82, 121, 145, 237, 243, 262, 263) and *in vivo* (76, 274).[7] All of the organisms examined for this aptitude have exhibited it; they include pneumococci, staphylococci, streptococci, meningococci, diphtheria bacilli, *Shigella spp.*, *Vibrio comma*, *E. coli*, gonococci, *Brucella abortus*, and *Hemophilus parainfluenzae*. In fact, it is the belief of some investigators that all organisms susceptible to the bacteriostatic action of the sulfonamides are capable of developing resistance to them (121, 262). Organisms have been trained to grow in concentrations even up to 0.018M (300 mg %) SA; this is, of course, with relatively small inocula. It must be emphasized, however, that no organism has ever been made totally resistant.

Because of its high clinical importance, the phenomenon of sulfonamide-fastness has received considerable attention. However, inasmuch as many of the issues involved are still in the controversial stage, only those regarded as essential to an evaluation of the theories proposed for the mode of action of sulfonamides will be considered here.

An extremely important feature of acquired "sulfonamide-fastness", especially from the clinical viewpoint, is that when an organism is made fast to one sulfonamide it is also resistant to the others (121, 145, 243). There have been cases reported in which acquired resistance is apparently not carried over from one sulfonamide to the others, but it is suggested by Kirby and Rantz (121) that such discrepancies are due to technical differences in the experiments, e.g., resistance to a sulfonamide may be missed if too high a concentration is used in the test. The latter authors have shown that the degree of resistance developed is correlated with the bacteriostatic potency of the sulfonamide; organisms made resistant to certain bacteriostatic concentrations of one sulfonamide are equally resistant to equivalent bacteriostatic concentrations of the others. This of course would seem to indicate, as Kirby and Rantz pointed out, that the acquisition of "fastness" represents an interaction between the bacteria and the one structural unit common to all the sulfonamides, namely, the *p*-aminobenzene nucleus, and, furthermore, that this interaction involves the same enzyme system as that concerned with PABA antagonism. Actual contact between the sulfonamide and the enzyme system involved, in fact inhibition of the same, appears to be prerequisite for the development of resistance, because PABA completely protects bacteria from such a change (262, 263).

The development of resistance in a strain is a gradual process (121, 237), developing most quickly to the least effective compound, and the more easily the less the original sensitivity of the organism (243).[8] Gonococcal colonies, for example, which develop after plating heavy suspensions on inhibitory concentrations of sulfonamide-agar are 5 to 10 or 50 to 100 times as resistant as the parent strain; and on further plating, occasional variants are obtained 1000 times as resistant as the original, thus approximating a logarithmic acquisition of resistance (145).

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[7] Acquisition of increased resistance to sulfonamides may not be confined to bacteria, for there is evidence that the flagellate *Polytomella* (164), *Endamoeba histolytica* (222), and macrophages (101) can develop increased tolerance.

[8] Resistance, as used here, refers to bacteriostatic concentrations of the sulfonamides.

The biochemistry, and especially enzymology, of bacteriology are still in their infancy; and it is difficult to get any insight into the problem at hand, namely, the reason why certain bacteria are greatly affected by certain environmental conditions while others are comparatively resistant, and what mechanisms the susceptible organisms have at their disposal to adapt themselves to unfavorable circumstances. The phenomenon of acquired resistance to a therapeutic agent is by no means new or confined to the sulfonamides, for it has been known for some time that the spirochete of syphilis can become "fast" to arsenicals, the pneumococcus to ethyl-hydrocuprein, and trypanosomes to various triphenylmethane dyes. Various possibilities have been proposed as to the mechanism of development of sulfonamide-fastness, and each has certain points in its favor. It may be that this phenomenon is more apparent than real, and that it merely represents a "weeding out" of the more susceptible bacteria, a true example of selection or survival of the fittest (181, 261). The fact that sulfonamide-fastness in a particular strain is a gradually developing process would be in agreement with such a hypothesis. That variability in sulfonamide susceptibility within a bacterial strain exists is now definitely established (238). Certainly, such a scatter of susceptibility to environmental influence in a presumably homogeneous population is a well-known phenomenon. Cases of bacterial adaptation without multiplication are known (48, 261) and it would, therefore, be of great interest to know if resting bacteria can develop sulfonamide-resistance. Such an approach may answer the question of the extent to which this phenomenon is a result of selection.

Schmidt and Sesler (236) utilized the other available method of approach to this problem, namely, a quantitative study of the sensitivity of individual organisms (pneumococci) composing a sensitive strain and the changes in sensitivity occurring during the strain's acquisition of resistance. It was found that the pneumococci present after the first exposure to SP were significantly more resistant than any organism in the original sensitive strain, and, within certain limits, organisms of increased resistance were formed on each additional exposure to sulfonamide. This indicates that resistant organisms are formed as a result of some action of the sulfonamide on the organism. These investigators emphasized that the individual pneumococci of a sensitive or resistant strain vary somewhat in their sensitivity, but that these differences are relatively small and not to be confused with the larger differences in sensitivity existing between the sensitive and highly resistant strains. They felt that although "breeding out" does not appear to be the main factor in the acquisition of increased sulfonamide-resistance, it has not been ruled out completely and that it probably does play a part. The question is whether or not the acquisition of drug-fastness is one of selection of hereditary variants which are specifically induced by the presence of the sulfonamide. Sulfonamides produce evolutionary changes in plants (B1b), and it may be that some type of hereditary mechanism is in operation also in bacteria.

As will be discussed in section D1h, it has been proposed that the varied sulfonamide sensitivity of organisms in general is directly related to their ability to synthesize PABA (or sulfonamide-antagonist), or to the rate of its release from the organisms into the medium. However, the available data are too confusing to warrant any blanket subscription; this is an aspect of the general problem which must be analyzed more thoroughly. Certainly it is possible that this may be the answer to the question with some bacteria at least; but there is no a priori reason why the mechanism of acquiring resistance to a drug must be the same in every instance.

One very plausible hypothesis takes cognizance of the extreme adaptability which is inherent in bacterial organisms (48, 113, 181, 261). The bacteria may develop insensitiveness to sulfonamides by adjusting their metabolic reactions in such a way as to render unnecessary for growth that particular reaction (or reactions) which is ordinarily inhibited by sulfonamides and the functional integrity of which is ordinarily essential for growth (237). This change may involve either the utilization of a new substrate (which may be synthesized by the cell) in a reaction unsusceptible, or less so, to sulfonamide action, and which can replace the one whose metabolism is blocked in susceptible cells, or the development of new intermediate metabolic pathways as shunts which bypass the susceptible reac-

tion. Such possibilities apply whether sulfonamide action is due to inhibition of anabolic reactions or inhibition of oxidative enzyme systems. Karström (113) has described variants with new enzymatic properties due to "adaptive" enzymes whose development and specificity are guided by the chemical structure of the substrate. These "adaptive" enzymes, however, are not permanent since they are lost when the specific conditions responsible for their development are removed; acquired sulfonamide-resistance, on the other hand, is usually retained long after the sulfonamide is removed (121, 180, 237).

A critical examination of the various enzyme systems of bacteria before and after development of sulfonamide-resistance should contribute much to the solution of this important problem.

7. Synergism with Specific Antibodies and Bacteriophage

There have been numerous reports of synergistic or potentiating action between sulfonamides and specific antibodies, both *in vivo* and *in vitro*. That their similar effect is attained through dissimilar mechanisms is borne out by three facts: first, PABA has no antagonistic effect on antibody action (264); second, acquisition of sulfonamide-fastness does not alter the susceptibility of bacteria to antisera (180); third, a bacterial strain may be resistant to serum and sensitive to sulfonamide, resistant to sulfonamide and sensitive to serum, sensitive to both, or resistant to both (19).

Sulfonamides also synergize and increase the lytic activity of bacteriophage (290) and lysozyme (207), in contrast to most antiseptics which inhibit or destroy bacteriophage. Though it is possible that some direct connection may exist between the action of sulfonamide and the action of antibodies or phage which would account for their tendency to summate their actions, it seems more probable that their combined effects represent a summation of unrelated influences unfavorable to the bacterial cell, i.e., they do not compete for the same receptor group in (or on) the cell.

Summary: To recapitulate briefly what has been given in some detail: sulfonamides inhibit not only the growth of bacteria but also the growth or other functions, or both, of numerous other cells; sulfonamide action, like the action of many other toxic substances, is usually biphasic; the activity is directly related to the sulfonamide concentration and to the temperature, inversely related to the inoculum size, influenced greatly by the structure of the sulfonamide itself and influenced by changes in pH. It varies not only from bacterial species to species, but even from strain to strain; and under certain conditions the relative activities of various sulfonamides can vary from bacterial organism to organism; bacteria can be trained to resist sulfonamides to a surprising extent; sulfonamide inhibition is synergized by antibodies and bacteriophage. This summarizes what is known about the relationships existing between the sulfonamide, the cell, and the environment. It does not tell us how the sulfonamides effect their inhibitory action.

C. ASSUMPTIONS THAT THE ACTION OF SULFONAMIDES IS DUE TO THEIR TRANSFORMATION INTO CHEMOTHERAPEUTICALLY ACTIVE FORMS

With this section begins consideration of the mechanism of sulfonamide action. Actually, the first part of this is not concerned with the mode of action, but with the question of whether or not the sulfonamide as such is the active agent. The idea that sulfonamides must first be oxidized before attaining activity is the foundation of two theories of the mode of action of sulfonamides, and therefore, must be described before considering the theories.

The material included in this section could perhaps have been dispensed with briefly in the introduction along with the other early proposals which have been outmoded, since it is generally conceded today that the amino-sulfonamide needs undergo no transformation to be bacteriostatic, it being the active form *per se.* The evidence for this is so conclusive that no lengthy, documented consideration will be given here. Because of the possible bearing on certain unsolved problems (sulfonamide toxicity, potentiation by oxidizing compounds, etc.), the author believes it worthwhile to outline the highlights of the earlier theories of sulfonamide action based on the assumption that a sulfonamide must first undergo some oxidative transformation before becoming active.

1. Oxidation of p-aminobenzenesulfonamide (SA) to p-hydroxylaminobenzenesulfonamide (HOSA)

Early in sulfonamide research the question arose as to whether *p*-aminobenzenesulfonamide (SA), as such, is the active agent. Mayer and Oechslin in 1937 put forth the hypothesis that SA is oxidized in the animal body and *in vitro* to *p*-hydroxylaminobenzenesulfonamide (HOSA), the substance responsible for the chemotherapeutic and bacteriostatic property. The finding of methemoglobin in the blood of animals and patients receiving SA, and in blood cultures containing SA, had suggested the formation of an oxidized product of the drug which was responsible for the oxidation of hemoglobin. SA, *per se*, is not an oxidant and is, therefore, incapable of such oxidative action, whereas certain oxidation products (exact nature unknown) of SA have been shown to be capable of such action.

The action of sulfonamide-antagonists, which will be considered in the next section, is difficult to fit into this hypothesis. Perhaps the greatest single piece of evidence against it is that PABA is unable to antagonize the action of SA oxidation products *in vitro* (83, 84, 223). Since it is well established that PABA can completely antagonize the therapeutic action of SA, it follows that only bacteriostatic substances which are antagonized by PABA have a true SA-like mode of action.

It is true that HOSA *in vitro* is more active than SA with some bacteria. HOSA *in vivo*, however, has proved to be no more active than SA, undoubtedly because of the great instability of HOSA, which is reduced to SA in blood very promptly. Proponents of this theory were of the opinion that HOSA is formed from SA slowly in the immediate vicinity of the bacterium, perhaps by the tissue cells of the host as well as by the bacterium itself, and probably with the aid of ferrous iron catalysis. Although it is conceded today that this is not the mechanism of sulfonamide action *in vitro* or *in vivo*, the fact, that oxidation products of sulfonamides, in certain cases at least, are much more active than their reduced forms, may offer certain therapeutic possibilities. Their instability precludes their use in any circumstances except where their oxidized state can be maintained by imposed oxidizing conditions, e.g. in local wound therapy.[9]

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[9] Neter (206, 208) observed that azochloramide potentiates the action of sulfonamides *in vitro*, whereas other chemotherapeutic substances such as optochin, merthiolate, and actinomycin do not even exhibit synergy (206). Schmelkes and Wyss (234) confirmed this azochloramide potentiation and, ruling out the possibilities that it might be due to a chemical reaction between the agents resulting in the formation of a more toxic compound on the grounds of the molecular ratio of the agents employed, and that it might be a result of a lowering of the sulfonamide threshold of the bacteria on the basis that the other compounds used by Neter produce no such effect, offered as a possibility the inactivation of sulfonamide-antagonists. Azochloramide and other chlorine compounds were found to inactivate PABA and peptone reversal of SA action on *E. coli*.

In the case of azochloramide potentiation, it is possible that the antagonist PABA is chlorinated and thereby loses its power of antagonism, for Wyss *et al.* (288) have found that 2-chloro-PABA and 3-chloro-PABA do not antagonize SA action.

In the light of some recent findings, another possible explanation of sulfonamide potenti-

2. Anti-catalase Theory

Based on the assumption of HOSA formation from SA *in vivo* and *in vitro* the "anti-catalase" theory was formulated, according to which, the therapeutic or bacteriostatic effect of SA is brought about indirectly by the following chain of events: *a*, SA is oxidized by the bacteria to HOSA; *b*, the latter inhibits catalase;[10] *c*, hydrogen peroxide resulting from bacterial metabolism, normally decomposed by catalase, now accumulates; *d*, when sufficient peroxide accumulates the bacteria are destroyed or their growth is hindered.

As stated, the biological evidence against the anti-catalase concept of the therapeutic action of sulfonamides is rather conclusive. Among the arguments which refute it are: *a*, Some bacteria are sulfonamide-sensitive in the absence of catalase; *b*, Type 3 strains of hemolytic streptococci which produce no detectable peroxide are susceptible to sulfonamide; *c*, certain peroxide-resistant organisms are sulfonamide-sensitive; *d*, certain bacteria are as sensitive to SA *in vitro* as to HOSA; *e*, PABA cannot antagonize the action of HOSA; *f*,

ation by azochloramide is conceivable, namely, that the potentiation is due to formation of HOSA or some other oxidation product of SA (this, of course, assumes that SA does not ordinarily undergo any oxidative change into an "active" agent). In the first place, Goldberger (80), in rather extensive experiments both *in vitro*, and *in vivo* by local application in infected wounds, found that oxidizing agents in general (Lugol's solution, azochloramide, dichloramine-T, hydrogen peroxide, zinc peroxide, potassium permanganate, etc.) potentiated sulfonamide action, while substances such as merthiolate and mercurochrome, did not. This potentiation was also shown in the effect on other unicellular organisms such as protozoa, spermatozoa, and certain fungi. Neter (209) and Crile (38) have confirmed Goldberger's observation that azochloramide enhances the activity of sulfonamide in localized infections. Other previous reports had claimed that hydrogen peroxide increases the effectiveness of local sulfonamide therapy. In the second place, as already stated, PABA is unable to inhibit the action of oxidation products of SA, for instance, HOSA. This would explain the observation that azochloramide "inactivates" PABA antagonism of SA activity. As already stated, the bacteriostatic action of such oxidation products is manyfold that of SA, at least on certain bacteria (84).

It is somewhat difficult to determine which hypothesis is correct. On the one hand, if the sulfonamide-antagonist is PABA it might be expected that this substance would be oxidized before any sulfonamide present. As a matter of fact, it has even been proposed that at least some of the sulfonamide-antagonistic action of PABA is a result of the latter being more easily oxidized than the sulfonamide (this assumes that the active drug agent is an oxidized product of the sulfonamide) (191). As will be seen in the next section, however, oxidized derivatives of PABA have been found to counteract sulfonamide action. Furthermore azochloramide destroys the reversing power of peptone, and there is evidence that the antagonistic activity of peptone is not due to its PABA content (section D). Acting on the suggestion that azochloramide may potentiate sulfonamide by oxidizing the latter, Sevag (244) studied a simple mixture of the two substances in the Warburg respirometer and observed no evidence of an oxidation. This, however, was in the absence of any iron or living material, and it is known, for example, that peroxide with ferrous iron catalysis is a rapid oxidant of SA (252). It may be that neither proposal is correct; but now that media devoid of sulfonamide-antagonists are available it may be that this problem can be solved.

[10] Hydroxylamine and oximes in general are known to inhibit catalase; actual inhibition of this enzyme by HOSA has also been adequately demonstrated. SA itself has relatively little anti-catalase activity (25, 36, 72, 184). This hypothesis was not based on an oxidative action of the oxidation derivatives of SA as was the original idea of Mayer and that of Shaffer and others, (this latter was concerned with the poising of the redox potential and will be considered presently), but rather on an anti-enzymatic action analogous to the inactive complex formation of carboxy-hemoglobin.

sulfonamides act in the absence of conditions necessary for the production of peroxide: e.g., certain anaerobes are susceptible *in vitro* and *in vivo*, and certain facultative organisms are susceptible under anaerobic conditions *in vitro*; *g*, HOSA can completely inhibit the aerobic respiration of streptococci and pneumococci (248). When respiration is completely inhibited there can be no formation of peroxide; a catalase inhibition under such conditions could not allow the accumulation of toxic amounts of a substance which is not being produced in the first place.

Thus one can conclude that while certain toxic and side effects of the sulfonamides may be caused by small amounts of oxidation products formed *in vivo* (102), and that under certain conditions with some organisms catalase inhibition may play a part in bacteriostasis, the principal mode of sulfonamide action both *in vivo* and *in vitro* is by some mechanism other than inhibition of catalase. It is true that the oxidation products exert an inhibition of bacterial growth *in vitro* but apparently this is by a different mechanism than the inhibition produced by the reduced compounds. This is further supported by the finding that *m*-nitrobenzenesulfonamide is as active as the *p*-nitro compound (223), a relationship certainly not existing (except under certain conditions to be discussed later) between the amino isomers. This mechanism would not necessarily have to be connected with catalase in any way.

In line with the discovery that PABA is unable to antagonize the action of sulfonamide oxidation products and the possibility that some toxic reactions *in vivo* may be due to such compounds, it has been shown that PABA is unable to inhibit sulfonamide rashes and fevers or acute toxic effects (167, 266). This, however, must not be regarded as too significant, because the actual mechanisms of sulfonamide toxic reactions are not yet understood; it has been recognized for some time that some of the toxic reactions are an allergic response.

3. The Poise of Oxidation-reduction Potential as Responsible for Sulfonamide Action

Once the hypothesis appeared that the active agent of sulfonamide action is some oxidation product of the parent compound, it was quite natural that interest should be aroused in the changes of oxidation-reduction potentials taking place in cultures during sulfonamide action. Investigation of such changes suggested that sulfonamides (in an oxidized form) might poise the E_h of of bacterial cultures at a level too high to permit bacterial multiplication.

Those who believed that sulfonamide oxidation products are the active inhibiting agents either were of the opinion that these products poised the potential at a high level because of their oxidizing power, or believed that the increased potential was a result of peroxide accumulation in the culture. Roblin and Bell (221) concluded from their experiments, however, that the high potentials obtained *in vitro* are due to the oxidizing agents employed in the determination, rather than to any SA oxidation products. This, of course, would not rule out the possibility that, in the absence of added oxidizing agents, sulfonamide bacteriostasis is accompanied by a high culture potential. However, the observation that the electrode potential of cultures in the state of sulfonamide bacteriostasis remains high can be argued from both sides. On the one hand, it can be claimed (as above) that the presence of oxidizing substances in the culture prevents the metabolic reactions required for growth. But on the other hand, it is possible that the metabolic reactions are hindered primarily, thus preventing the decrease in potential observed during growth and which probably is a natural result of bacterial metabolism (94). The fact that the active sulfona-

mide is now known to be not in an oxidized state definitely indicates that the latter possibility would explain the high potentials if actually present during sulfonamide action.

D. SULFONAMIDE-ANTAGONISTS[11]

It was called to the reader's attention very near the beginning of this review that one of the most unusual facts about sulfonamide action is that certain substances can completely counteract it, and frequent reference to this phenomenon has already been made. The phenomenon of drug antagonism was not wholly unknown previously but this observation was so clear cut, and it came at a time when everyone was so anxious to get at the secret of sulfonamide action, that immediately it stirred the whole sulfonamide field to the point that nearly all the research in the field in the last few years has been devoted to the study of sulfonamide-antagonists. As a result of these investigations several theories of sulfonamide action have evolved, and in order to understand and critically view these theories, it is necessary to present in some detail what is known about sulfonamide-antagonists and their actions. The theories themselves will be developed and discussed as an integral part of this presentation.

1. Para-aminobenzoic Acid (PABA)

The sulfonamide-antagonists can be conveniently divided into two groups: antagonists of known composition, and antagonists of unknown composition which for the main part are mixtures. One of the theories which arose as a result of the study of antagonists is the Woods-Fildes theory, which, since the time of its appearance in 1940, has enjoyed practically universal subscription by the investigators in the field. The Woods-Fildes theory gained this support because it correlates very neatly some of the major observations of sulfonamide action. Since its appearance, many observations have been found compatible with the theory and these have served to make its general acceptance more nearly complete. However, several other important observations made in this same period of time have found no place in this theory and to a great extent have therefore been ignored. It is timely that a critical examination of this theory be made. The Woods-Fildes theory, as well as one other to be discussed, is based on the action of the antagonist, PABA. Before attempting an interpre-

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[11] Apparently it is becoming customary to call these substances "sulfonamide inhibitors". It is suggested that to avert confusion the term "inhibitor" be retained in its original connotation, that of the inhibitor of a cell function or reaction, and that such a term as "antagonist" be applied to substances which prevent the action of inhibitors.

Sulfonamide-antagonists have been called "anti-sulfonamides" by some, their action being called "anti-sulfonamide action". In the field of immunology the prefix "anti-" before a substance connotes that an "anti-substance" has been produced as a response to an antigen and can combine with that antigen. It is best that the term "anti-sulfonamide" be reserved for such use.

"Reversal of sulfonamide action" has also been frequently referred to in the literature. This as used has seldom meant that sulfonamide action existed which was subsequently reversed, rather in most instances it has meant that the sulfonamide action was prevented from ever developing. Although this is a point which is presumably only of importance as a matter of terminology, the use of the term "reversal" has been avoided in this review.

tation of the mechanism of this antagonism, it seems proper to consider all that is known about PABA and its properties.

a. Distribution and isolation. The first experimental indication that there may be a fundamental SA-antagonist appeared in the work of Woods (284) in which it was shown that yeast extracts contain a sulfonamide-counteracting factor whose chemical properties suggest a close chemical relationship to SA itself. PABA was found to have high activity in sulfonamide-antagonism, and it was suggested that the sulfonamide-antagonist in yeast might be PABA. Rubbo and Gillespie (226) believed they had obtained the benzoyl derivative of PABA from yeast. Isolation and chemical characterization of PABA from yeast was reported by Blanchard in 1941 and subsequently by others (14, 174, 228)[12]. PABA was obtained both in a free and in a combined form. The latter, Blanchard suggested, may be the sulfonamide-antagonist which Loomis *et al.* (161) had obtained from yeast, and may be the substance from which PABA is derived when yeast is autolyzed. The exact nature of this combined form is obscure, the substance obtained by Loomis *et al.* (161) was insoluble in ether, but it may be a peptide (14); non-diazotizable, and not inactivated by acetylation, whereas the physical and chemical properties of PABA are exactly the opposite. Green and Bielschowsky (83) also found evidence of an ether-insoluble SA-antagonist in their bacterial extracts.

Part of the antagonist obtainable from bacteria (83) and plasma (174), and practically all contained in normal human urine (179) is in a conjugated, inactive form which becomes active only following hydrolysis. It appears that the antagonist is conjugated in the body and excreted in the urine in this inactive form. These antagonists may be PABA but they have not been characterized. It may be interesting to note in this connection that PABA fed to man or animal is excreted partly as its acetyl derivative and perhaps as a glucuronate (92).

Sulfonamide-antagonists have been found in many diverse places, but as will be seen, whether or not they are PABA has not been definitely established except in yeast. It has also been suggested that, in all organisms other than bacteria in which PABA counteracts sulfonamide inhibitory activity, the PABA plays a part in the normal metabolism (probably as an essential metabolite) of the particular sulfonamide-susceptible organism. There is no experimental evidence for this postulate; the possible "wide distribution" of PABA in the plant and animal kingdoms is still an open question.

b. As Sulfonamide-antagonist. Woods and Fildes (284, 285) first demonstrated PABA antagonism of sulfonamide inhibition of bacterial growth. This antagonism to the action of all the sulfonamides on bacteria *in vitro* has been confirmed by many workers under very wide experimental conditions (75, 83,

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[12] Landy and Dicken (140), Lewis (153), and Mitchell *et al.* (200) found that of all substances assayed for PABA by their microbiological methods yeast is the richest source. Substances assayed and found to contain PABA included liver, spinach, oat seeds, mushrooms, meat extract, urine, blood, and peptone. These microbiological assay methods depend on the growth-factor specificity of PABA for *Acetobacter suboxydans, Lactobacillus arabinosus,* and a strain of *Neurospora crassa,* respectively.

91, 179, 227, 286); in fact, to date no single report denies the counteraction of sulfonamide action on bacteria by PABA. PABA antagonism is also manifest *in vivo*. In mice, PABA has been shown to antagonize SA, SP and ST in hemolytic streptococcal infection (218), to antagonize SA, SP and ST in pneumococcal infection (167), SD in meningococcal infection (218, 270), and ST in infection by *Klebsiella sp.* (205).

PABA has been found to antagonize sulfonamide action in experimental malaria (182, 188, 240); in experimental lymphogranuloma venereum virus infection (62); on a fresh water diatom (278) and algae (30) (autotrophic plants in comparison to pathogenic bacteria which are heterotrophic); on *Pisum* seedlings (279); on *Pisum* and *Lupinus* rootlets (183); on tomato roots (16); on a dermatophyte (43); on *Neurospora crassa* (268); on *Aspergillus niger* and the flagellate *Polytomella caeca* (164); on yeast (139); on luminescence in growing cultures of luminescent bacteria (105); on pigment formation by *Pseudomonas aeruginosa* (271). The antagonistic activity of PABA varies considerably with different organisms.

The relationship existing between sulfonamide and the antagonist PABA is a competitive one as has been shown by Wyss (286) and by Wood (281) using *E. coli*. In the presence of PABA the growth-stimulating concentration of SA is increased (136), and, over a wide range of concentrations, the ratio of sulfonamide to the amount of PABA required for antagonism is more or less constant (84, 173, 205, 227, 269, 281, 284, 287); these two observations are to be expected from the mass law relationship and, therefore, they offer further support for such a relationship. It has been found that one mole of PABA will counteract approximately 1,000 to 26,000 or more moles of SA (43, 70, 75, 163, 226, 231, 284, 287). There have been reported instances in which this ratio is nearer unity (133, 169, 246, 286). Much of the responsibility for this extreme variability in the ratio probably lies in the variable amounts of sulfonamide-antagonists already present in practically all culture media. The other sulfonamides under the same experimental conditions require relatively more PABA to counteract their action (70, 75, 84, 115, 205, 246, 281, 284, 287). Analysis of the quantitative data shows that the potency of each sulfonamide is directly proportional to its ability to counteract the anti-bacteriostatic action of PABA, in other words, the amount of PABA required to counteract bacteriostasis is approximately the same for the minimal bacteriostatic concentration of each sulfonamide (75, 128, 281, 284). This is to be expected from the law of mass action (124). As has been pointed out by several investigators (173, 227, 281), such a large ratio as one mole of PABA to 1,000–26,000 moles of SA does not rule out competitive inhibition between the sulfonamide and PABA; it could be that the relative affinities are widely separated. These high molar ratios, however, are figured from data obtained in a pH range where practically all the SA activity is a function of the SA-ion, and if the ratio of SA-ions to PABA is used to represent the actual competitors in the reaction, values much nearer unity are obtained (75).

Other substances closely related to PABA, procaine for example, also show this phenomenon of sulfonamide-antagonism. According to Woods (284) the

action of procaine is slightly delayed, and it may be, therefore, that hydrolysis of the ester is necessary before it becomes active (179, 228, 284). Similarly p-nitrobenzoic acid, p-hydroxylaminobenzoic acid, p-aminobenzamide, p-amino-benzaldehyde, and p-nitrobenzaldehyde may owe their antagonistic action to their conversion to PABA (196, 218, 227, 228, 284). Not only procaine, but other local anesthetics derived from PABA counteract sulfonamide action in vivo and in vitro, whereas others not derived from PABA are devoid of activity (115), and as Krahl points out (130) it would be very difficult to prove or disprove that the PABA derivatives owe their activity to hydrolysis to PABA. Johnson et al. (107) obtained evidence indicating that the inhibitory action of procaine on bacterial luminescence is not due to its hydrolysis to PABA; inhibition by procaine was counteracted by increased pressure, whereas pressure had little or no effect on the inhibition caused by PABA. It is of interest to note that p-nitrobenzoate in sufficient concentration is itself capable of growth-inhibition which is reversed by the further addition of a small amount of PABA (196). This certainly indicates that the p-nitrobenzoate molecule does not have to be reduced in order to have an affinity for the same locus at which sulfonamide inhibition occurs.

Recently, Auhagen (4) found that p-aminobenzoyl-l-glutamic acid is 8 to 10 times more active than an equimolar concentration of PABA in counteracting in vitro the SA-inhibition of Streptobacterium plantarum. The p-aminobenzoyl derivatives of d-glutamic acid, l-aspartic acid, l-leucine, d-leucine, glycine, and glycyl-glycine were inactive. The antagonistic activity of p-aminobenzoyl-l-glutamic acid obviously cannot be a result of transformation to PABA.

Comparison of properties of the isomers of PABA and SA. The structural similarity between the active sulfonamides and the antagonist PABA has been stressed as a fundamental basis for their mutual competition for an enzyme surface (12, 60, 91, 173, 227, 281, 284). This raises the question of the activity of the ortho- and meta-isomers of PABA. Rubbo and Gillespie (226, 227) found that PABA is approximately 10,000 times more active than either its ortho- or meta-isomer as a growth factor for Clostridium acetobutylicum, and suggested that the very small activity of the isomers may be due to PABA impurities in the preparations. With respect to sulfonamide-antagonistic activity, however, PABA was only 5 times as active as the same preparation of meta-isomer. This throws considerable doubt on the suggestion that the activity is due to PABA as an impurity; and it would seem, therefore, to indicate that the isomer can counteract sulfonamide action, though less actively than the para compound. Using concentrations required for counteraction of sulfonamide as a criterion of activity, others have reported that the ortho- and meta-isomers are antagonistic, although their activity in this respect is much less than that of PABA (284). There have been a few reports of failure to demonstrate any sulfonamide-counteracting activity of the isomers; in some of these reports, the concentrations used are not stated, and in the others the concentrations used were below those at which others have observed the antagonistic activity. The ortho-isomer has been shown to antagonize the therapeutic action of SP on mice infected with

pneumococcus or *Streptococcus pyogenes*, though much less so than PABA (218). It is significant, however, that in these experiments the same amounts of the two isomers were administered.

The isomers have also been reported as unable to replace PABA as growth factor for *Acetobacter suboxydans* (140), and the fungus *Neurospora crassa* (268). Lewis (153) reported the *ortho-* and *meta-*isomers to have 0.00005 and 0.009 per cent, respectively, of the growth-stimulating activity of PABA for *Lactobacillus arabinosus*, and expressed the belief that even this relatively weak activity may be due to PABA-impurity in the compounds. The *ortho-*isomer, however, is capable of replacing tryptophan as a growth essential for *L. arabinosus* and *L. casei*, but not for various other lactobacilli (254). This activity is not inhibited by orthanilamide in a concentration 10,000 times that of the *o*-aminobenzoic acid. This fact is insufficient to rule out the possibility of a competition due to structural similarity, since higher concentrations of orthanilamide might have produced an inhibition.

Although definite proof in the form of critical quantitative data is lacking, it would seem probable that the isomers of PABA do possess properties similar to PABA, and that the differences in activities (as growth-factor and sulfonamide-antagonist) of the isomers are due to differences in adsorptive affinities for the same locus.

Then, too, there is the question whether the isomers of SA possess any true SA-like activity. Early reports claimed them to be inactive, but with one exception failed to state the concentrations employed; Nitti *et al.* (210) found them to be inactive in the concentration 0.006 M (100 mg %). That inactivity of the isomers could not be a result of a lack of adequate blood concentrations, nor to a failure of the compounds to adsorb on, or diffuse through, the bacterial cell was shown by Feinstone *et al.* (56, 57). In electrokinetic experiments Bradbury and Jordan (18) observed that metanilamide behaves like aniline at the bacterial surface, not like SA or PABA. These authors believed that the activity of the *para-*compounds is due to polar resonance which, of course, is impossible with the *meta-*isomer, but they were at a loss to account for the inactivity of orthanilamide, since this compound is capable of resonance. Kumler and Daniels (134) explain this apparent discrepancy by assuming a hydrogen bond to exist between the amino and the sulfone groups; the amino group is therefore not free, a structural prerequisite for activity (cf. B3g).

Although it is generally accepted today that the isomers of SA are inactive, actually there is very little critical experimental evidence to support this viewpoint. Wyss *et al.* (289) obtained greater respiratory inhibition of several bacteria with orthanilamide than with either the *meta-*compound or SA, the latter two inhibiting to the same extent. These experiments were carried out in a synthetic medium devoid of sulfonamide-antagonists. Sevag *et al.* (245) have found that orthanilamide exerts a greater inhibition on the carboxylase activity of *E. coli* than SA in the same concentration, metanilamide a lesser inhibition. The important observation, however, was that peptone, serum albumin, and globin at any particular concentration antagonize the inhibitions by the *ortho-*

and *meta*-isomers to a very much greater extent than that by SA. The possible explanation of this will be discussed later, but suffice it to point out here that this observation could very well explain the inactivity of these compounds *in vivo* where sulfonamide-antagonists are ever present, and *in vitro* when media containing antagonists are employed. It may be, therefore, that although the isomers are of much less or perhaps no value therapeutically, they have been too hastily regarded as having no fundamental action akin to SA. Then too, the mere fact that they are inactive at the concentrations in which SA exerts its effect does not rule out the possibility of their acting similarly at higher concentrations.

Actually, there is a question whether structural dissimilarity does rule out competition for the same enzyme. McIlwain (171) noted the lack of specificity between certain bacterial inhibitors and their antagonists of corresponding structure. The phenomenon of adsorption in general does not necessarily imply that two substances adsorbing onto the same surface must be structurally related, and so long as the adsorption of each substance is reversible there will be a competition according to their relative affinities. For example, narcotics of entirely different molecular configuration can inhibit the same enzyme. Thus, Möller and Schwartz (202) report that PABA can counteract the inhibitory action of germanin, neostibosan, arsphenamine, and neo-arsphenamine (compounds devoid of any structural similarity to PABA) on *Streptobacterium plantarum*.

c. As essential metabolite.[13] Woods and Fildes (60, 284, 285) proposed the theory that sulfonamides function by interfering with an essential metabolite, and thus inhibit growth,—the essential metabolite being *p*-aminobenzoic acid. They further proposed that such inhibition requires an inhibitor closely related to the essential metabolite so that it can fit the same enzyme, but sufficiently unrelated to be an inadequate substitute for the essential metabolite. The Woods-Fildes theory thus was based on the existence of a competition between the sulfonamide and PABA for an enzyme surface, and as already seen, this competition has been amply confirmed.

Cases of competitive inhibition of enzyme reactions by substances related to substrates or products are well known (89): succinic dehydrogenase by malonic acid; lipase by acetophenone and other non-polar compounds containing a carbonyl group; lactate dehydrogenase by α-hydroxybutyric acid, glyceric acid, mandelic acid, hydroxymalonic acid, glyoxylic acid and oxalic acid; invertase by β-glucose, α- and β-fructose, β-*l*-arabinose, and α- and β-galactose.

Recent investigation has revealed other anti-bacterial agents which are related to growth essentials in the manner that SA is related to PABA and whose mutual specific effects may be readily explained in terms of competitive inhibition. Bacterial growth inhibited by the addition of certain sulfonic acids or their amides can be restored by adding corresponding carboxylic acids or their deriva-

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[13] An essential metabolite is a food substance essential to the organism but which the organism may be capable of synthesizing. A growth factor, on the other hand, is not only essential but must be supplied as such for the organism since the latter is unable to synthesize it.

tives (170). It is considered that the carboxylic compounds play an essential role in growth reactions which are interfered with at enzyme surfaces by the similarly constituted sulfonic compounds. The growth-inhibition produced by α-amino sulfonic acids can be reversed with α-amino carboxylic acids (171). When bacteria are made independent of added amino-carboxylic acids by training, the α-aminosulfonic acids lose their inhibitory power.

Sulfonic acid analogs of pantothenic acid (e.g., pantoyltaurine) are bacterial growth-inhibitors, their action being negated by concomitant addition of pantothenic acid (9, 253). Pantothenic acid is a growth factor for all organisms which are found susceptible to pantoyltaurine (175). Bacterial inhibition by pyridine-3-sulfonamide is unaffected by PABA or pantothenic acid, but is antagonized by nicotinamide (and nicotinic acid); inhibition by pantoyltaurine is antagonized by pantothenic acid but not by nicotinamide or PABA (173, 175). Pyridine-3-sulfonic acid inhibition is also completely antagonized by thiazolecarboxylic acid, coenzyme I, and "iron ion" (201). Pantothenic acid antagonizes pantoyl-taurine's chemotherapeutic activity *in vivo* as well as its inhibitory action *in vitro* (176). The antagonism of pantoyltaurine by pantothenic acid and the antagonism of pyridine-3-sulfonamide by nicotinic acid are both competitive, since as with PABA and sulfonamide, a constant ratio exists over a wide range between the inhibitor and the amount of antagonist required for counteraction (170, 175, 176). Snell *et al.* (225) report that long-continued administration of pantoyl-taurine to mice and rats produces evidence of pantothenic acid deficiency, thus indicating that this compound may interfere specifically with pantothenic acid metabolism in animals as well as in bacteria.

Indoleacrylic acid inhibits bacterial growth, and this action can be counteracted by the addition of even a trace of tryptophan (61). As Fildes stated (61), this latter observation, plus the fact that, in this instance there is complete absence of a competitive relationship between inhibitor and inhibitor-antagonist, suggests that the action of this inhibitor is more likely to be concerned with the formation of tryptophan rather than its use. There should be a quantitative relationship between indoleacrylic acid and some precursor of tryptophan.

This, as Fildes (61) emphasized, calls attention to a rather important point, namely, if sulfonamide action were a case of inhibiting PABA-synthesis, it would not be expected that the inhibitor should have a quantitative relation with the product PABA but with a precursor, and furthermore, the addition of PABA just sufficient for the needs of the organism should cause growth in spite of the presence of any amount of inhibitor, which of course is contrary to all observations. Others also have concluded that sulfonamide action is inhibition of the utilization rather than the synthesis of PABA (84, 163).

In view of the fact that PABA has been isolated from yeast, the effect of sulfonamides and its relation to PABA in this organism is of especial interest. Landy and Dicken (139) carried out a rather thorough investigation of the effect of sulfonamides (SA, SP, SG, and ST) on *Saccharomyces cerevisiae* in synthetic media. All of the compounds were found to inhibit yeast growth completely in concentrations ranging from 10 to 25 mg %. This inhibition was completely

counteracted by PABA, and it is of interest to note that the molar ratio: sulfon-amide/PABA in this antagonism compares very well with that reported for bacteria. It was discovered that the supernatant of 16-hour yeast cultures contains a substance which neutralizes the inhibition by ST. In view of these facts, Landy and Dicken believed it quite probable that the mechanism of sulfon-amide action on yeast is similar, if not identical with that on bacteria, and they suggested that PABA may be important in yeast metabolism as Woods and Fildes' "essential metabolite."

It is extremely difficult to interpret experiments involving the use of drug-antagonists, though this is one of the most promising approaches to the solution of the problem of drug action. There is danger in assuming prematurely that antagonistic agents characterized by the effects on growth are the compounds normally involved in the inhibited reactions. Moreover, the metabolism of different bacteria, even of different strains of some species, differs considerably, thus greatly complicating the picture. The fact that complex media may con-tain various antagonists cannot be ignored, and indicates that much may have to be learned from the use of such antagonists in synthetic media of known com-position. When bacteria are put in such artificial media they may be far re-moved from their optimal environment. Carrying over results thus obtained to the interpretation of drug action on the bacteria in the host must be done with caution. The evidence so far is confusing, and to make order out of chaos is not easy. It would appear, on the one hand, that the mechanism of sulfonamide action and its antagonism is far from simple. On the other hand, the fact that the potency of each sulfonamide is proportional to its ability to nullify the effect of PABA-antagonism, plus the fact that PABA counteracts all sulfonamides, suggest that bacteriostasis is produced by interference with a single metabolic function of the cell. In order to prove that PABA is connected with such a metabolic function in the role of an essential metabolite, it would be necessary to show that PABA is actually an essential metabolite, identify the enzyme system with which it is associated, and then demonstrate that the effect of sulfonamide is directly proportional to the inhibition of this enzyme system.

As will be seen in the next part of this section, certain bacteria require PABA for their growth. Certain investigators have expressed the belief that the re-quirement of PABA as a growth factor by these few bacteria indicates rather definitely that PABA plays a definite role (e.g., as an essential metabolite) in the metabolism of other bacteria in which PABA is not a growth factor. This supposition of course is used as an argument for the Woods-Fildes theory which presumes such a role for PABA in the cell. It must be remembered, however, that those bacteria which require PABA for growth are saprophytes and as such are not as exacting in their nutritional requirements as the pathogens. It is a curious fact that, to date, PABA has been claimed to be essential for the growth of only one of the pathogenic bacteria (diphtheria bacilli, cf. part d). Even in this instance, however, the pathogenicity of the particular strain used was not recorded. Hence, there is no justification for the generalization that PABA has a metabolic role in all bacteria merely because it is a growth-factor for some of

them. It must be admitted, however, that some of the bacterial products which will be mentioned subsequently may well be PABA, although if this were true it would not necessarily indicate that PABA is an essential metabolite. Landy *et al.* (141), assuming that the sulfonamide-counteracting substance they found to be produced by all pathogens assayed is PABA, expressed the opinion that it is unlikely that so many unrelated species of bacteria would all make this compound unless it is an essential metabolite. In instances where PABA is an essential metabolite but not a growth factor, it would be very difficult to prove this until more is known about the particular enzyme system involved.

As will be seen in the next section, PABA antagonizes the sulfonamide inhibition of carboxylase, an instance in which PABA can scarcely be claimed to be playing the part of an essential metabolite. This is even more true of PABA-antagonism of sulfonamide inhibition of starch digestion by diastase and of methylene blue adsorption onto charcoal (54). Thus, that sulfonamide inhibition is a result of displacement of PABA, functioning as an essential substrate, cannot be the only possible explanation compatible with the known facts. Other interpretations will be presented in section F1.

d. As a growth factor.[14] Up to the time of Woods' and Fildes' publications (60, 284), PABA had been isolated from neither bacteria nor yeast, nor had it been proved to be a growth factor or essential metabolite. Shortly after these publications appeared, Rubbo and Gillespie (226) reported PABA to be a growth factor, as well as a SA-counteracting factor, for *Clostridium acetobutylicum*. Lampen and Peterson (137) and Park and Wood (216) were unable to reproduce Rubbo and Gillespie's experiments on PABA as a growth factor unless biotin was also present, and the latter authors expressed the belief that the substances (perhaps glucose) used by Rubbo and Gillespie contained biotin.

PABA has also been shown to be a growth factor for *Acetobacter suboxydans* (138, 140, 142) and probably for *Lactobacillus arabinosus* (100, 153). In the latter instance, lactic acid production is stimulated by PABA. It has also been claimed that PABA is a growth factor for *Corynebacterium diphtheriae* type *gravis* (29).

PABA concentrations in and above the range 1.5×10^{-5} M (0.2 mg %) to 0.01 M (140 mg %) have been reported by various investigators as growth-inhibitory (4, 84, 91, 226, 227, 248, 284). It has been noted (248) that the supposed growth function and the SA-counteracting action of PABA is thus restricted to a "zone of limited concentration", which is unusual for known bacterial growth factors. It would seem, however, that this consideration *per se*

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[14] Recent work by Eyster (55) reveals that growth substances can achieve their effects indirectly as well as directly. Eyster shows that auxins are growth substances because they bring about the release of certain enzymes such as diastase from an adsorptive combination with protein colloidal substances, in which form they are relatively inactive. A rather analogous situation may exist in the case of thiamin stimulation of cocarboxylase (diphosphothiamine) activity in sea urchin egg extracts (131). It is thought that the thiamin might produce this effect by displacing the cocarboxylase from a combination with catalytically inactive protein, thus allowing the cocarboxylase to combine with the catalytically active protein.

does not constitute a conclusive argument against the possibility of PABA being an intermediary metabolite, since practically any substance will inhibit cell metabolism beyond some concentration level.

As stated previously, the fact that PABA is a growth factor for some bacteria has been used as evidence for the Woods-Fildes theory. Let us now examine this evidence further. Bacteria susceptible to sulfonamides may be arbitrarily divided into two groups: one for which PABA is a growth factor, and the other for which PABA is not a growth factor. The assumption is made that the only difference between the two groups, insofar as the relationship of PABA to the metabolism is concerned, is that, in the one, sufficient PABA can be manufactured by the cell for its needs. Accordingly, when both types of bacteria are inhibited by a sulfonamide, the antagonism offered by the concomitant addition of PABA would be the same in both cases, i.e., when the sulfonamide inhibits the bacterium it does so by displacing PABA which is essential for growth; whether the bacterium is capable of manufacturing PABA thus has no direct relationship to the mechanism of sulfonamide inhibition. This assumption makes the inhibition of bacterial growth resulting from withholding PABA from a bacterium for which it is a growth factor analogous to sulfonamide inhibition of bacterial growth, no matter whether the bacterium inhibited by the sulfonamide requires PABA for growth or not. This is supported by the report of Wyss et al. (288) that the SA-counteracting activity of PABA parallels its growth-factor activity both for *C. acetobutylicum* and for the "aminobenzoicless" mutant of *Neurospora crassa.*[15] Actually, however, there is indication of a real danger in making such an assumption. That there may be dissociation between these two phenomena (growth-factor activity and sulfonamide-antagonistic activity) is suggested by the fact that although *p*-aminophenylacetic acid is ten times more active than PABA as a growth factor for *C. acetobutylicum*, it possesses no SA-counteracting activity with the same organism (228). Nor does *p*-aminophenylacetic acid have any sulfonamide-antagonistic activity for other organisms (163).

[15] Tatum and Beadle (268) were able to produce an X-ray induced mutant of *Neurospora crassa* which is characterized by the loss of ability to synthesize PABA. A single gene is involved in this mutation, the gene apparently controlling an essential step in the synthesis of PABA. Growth when PABA is supplied is indistinguishable from that of the normal strain, but the mutant is unable to grow on synthetic media devoid of PABA (or the less active substances acetyl-*p*-aminobenzoic acid, *p*-nitrobenzoic acid, aniline and a few others). A concentration of 0.006 M (100 mg %) SA inhibits the growth of both strains to the same extent and in both cases this inhibition is counteracted by PABA. Since the quantitative effects of SA were the same on the two strains it was concluded that PABA utilization is interfered with rather than its synthesis. Results also indicated that the synthesis of PABA in the normal strain does not involve the introduction of an amino group into a preformed benzene ring, the nitrogen or amino group probably being incorporated before the ring is formed. The similarity in the action of SA and PABA in this particular instance seems especially significant inasmuch as it has been demonstrated that PABA (also pyridoxin and pantothenic acid) is involved in the respiratory mechanism of *Neurospora* (79). When mutants are used which require one of these substances for growth, the respiration of the mutants in media containing adequate substrate, but deficient in the particular "growth-factor," is increased upon the addition of the factor.

e. As vitamin.[16] A vitamin is a substance which the animal body requires for normal growth or other normal function, and which the organism itself cannot synthesize. Thus, fundamentally, the terms "growth factor" as applied to bacterial nutrition and "vitamin" as applied to animal nutrition are analogous. The suggestion that PABA occupies an integral position in bacterial nutrition, and the belief of some investigators that PABA is widespread in the animal and vegetable kingdoms make the question of its effect on the animal organism of considerable interest.

PABA has been tentatively added as a member of the vitamin B complex on the basis of its being essential to reproduction and lactation in rats, and growth-promoting in chicks. Subsequent studies have revealed real discrepancies in such an interpretation of the role of PABA in animal nutrition. Recent evidence suggests that PABA is not a growth factor for chicks, but that its action may be indirect by stimulating intestinal bacteria to produce certain essential factor(s) (22).

It is not safe at present to conclude that PABA *per se* behaves in any instance as a vitamin. Although this by no means excludes the possibility that PABA is present in the animal organism, it removes for the present at least one source of support for the idea.

f. Its relation to the development and prevention of resistance to sulfonamides. In the discussion on the acquisition of sulfonamide-resistance (B6) it was stated that one of the explanations offered for this phenomenon is that the bacteria increase their production of PABA, so that a correspondingly larger amount of sulfonamide is required for inhibition (82, 83, 84, 143, 163). This is a logical extension of the Woods-Fildes theory. The establishment of its validity depends on a direct demonstration of an increased production of PABA in the sulfonamide-resistant strain as compared to its parent strain, and furthermore, that this increased production is proportional to the amount of resistance developed.

There have been several reports that the acquisition of resistance is accompanied by an increased production of sulfonamide-antagonist (82, 83, 179, 198). This antagonist was believed to be PABA either on the mere fact that the substance was a sulfonamide-antagonist, or on the fact that the substance was destroyed by a soil bacillus trained to oxidize PABA (198),[17] or on the basis of certain physical and chemical properties of the substance, such as solubility and diazotization (143, 198), or on the basis of microbiological assays (143, 228). The observation that *S. aureus* when grown in increasing amounts of sulfonamides produces a yellow pigment believed to be a PABA derivative has also been considered as evidence that sulfonamide-resistance is a result of increased PABA production (258).

Green and Bielschowsky (83) were among those who observed that resistant strain washings have a greater sulfonamide-antagonistic action than those of the parent; but they found no strict correlation between sensitivity and yield of sulfonamide-counteracting factor, and thus came to the conclusion that a complete explanation of acquired (and natural) resistance cannot be put solely on such a basis. Landy *et al.* (143) found that sulfonamide-resistant strains of

[16] See György (88) for a review of this subject.
[17] The antagonist also specifically activated the PABA-oxidizing enzymes of the soil bacilli grown in its presence. Inasmuch as growth took place during this process, it would appear that PABA could not be an essential metabolite for these bacteria; yet this particular organism is sulfonamide-sensitive (199).

S. aureus produce greater amounts of PABA than do their parent strains (supernatant assayed by the microbiological test of Landy and Dicken, and by chemical methods), and that the amount synthesized by the resistant strains appears sufficient to account for their resistance. On the other hand, resistant strains of *E. coli, Vibrio comma, Shigella dysenteriae,* and pneumococcus failed to synthesize greater amounts of PABA than did their parent, non-resistant strains. The possibility of other, unknown sulfonamide-counteracting substances being produced in greater quantity by the resistant strains was not ruled out in this investigation.

The results have been inconsistent. In the first place, although in many instances there has been an increased production of sulfonamide-antagonist accompanying the development of resistance, the methods used for identifying the substance as PABA can be questioned. (This particular criticism will be given in greater detail subsequently.) Moreover, it is not even established that there is a strict parallelism between the production of any sulfonamide-antagonist and resistance, perhaps because the mechanism of acquiring resistance to sulfonamide is not identical in every case. Thus, the prerequisites stated at the beginning have not been satisfied. Although it must be admitted that the possibility has not been ruled out that sulfonamide-resistance in some instances is a result of an increased production of sulfonamide-antagonist (perhaps PABA), it must be concluded at present that this cannot be the mechanism in every case, and that it has not even been definitely shown to be the mechanism in any case.

Actual contact between the sulfonamide and the enzyme system involved, resulting in inhibition of the same, appears to be essential for the development of resistance (121, 262). Thus, PABA completely prevents the bacteria from developing such resistance; methionine, nucleic acid, and peptone, on the other hand, delay acquisition of resistance but do not prevent it (262, 263). This cannot be used as supporting evidence for the mechanism for the evolution of sulfonamide-fastness discussed above. It seems reasonably certain that antagonists achieve their action by causing removal in some way of the sulfonamide from its site of action. Since it is logical to assume that resistance develops as a protective response on the part of the organism to the stimulus of the toxic substance sulfonamide, it follows that, unless the toxic state exists, resistance will not develop.

g. As "catalyst" related to the synthesis of substances such as methionine. In the Woods-Fildes theory PABA is considered to be a substrate. Kohn and

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[18] Although these publications of Kohn and Harris will be referred to several times, it seems best to mention at this point that the pH fell in the course of their experiments, in some instances as low as 4.7 (126). In view of the established fact that sulfonamide activity varies considerably with pH, and the definite possibility that the activity of sulfonamide-antagonists may also be affected, quantitative results and their interpretation in these publications can be seriously questioned. The theory proposed, however, should receive consideration here inasmuch as it was partly based on previously established facts, and furthermore, because the essence of the theory, that sulfonamide inhibition is primarily on the synthesis of the essential food substance, has received verbal support elsewhere (241, 281).

Harris (91, 126)[18] formulated a new theory which transferred PABA from the role of a substrate to that of a catalyst. The essential facts underlying this interpretation are the following: First, as already discussed, many workers have reported that a latent period exists before sulfonamide action manifests itself in *vitro*. Second, methionine is able to counteract sulfonamide action, but only at low concentrations of sulfonamides. Third, ethionine, norvaline, and norleucine inhibit bacterial growth and synergize SA-action, possibly by competing with and displacing methionine in the cell, since addition of methionine (or peptone), but not PABA, abolishes these inhibitions. It is as if these compounds endeavor to take the place of methionine in a reaction normally involving methionine but are an inadequate substitute for the methionine; thus the reaction ceases. The fact that PABA does not affect inhibition by ethionine was regarded as placing the methionine antagonism as a reaction secondary to primary reactions involving PABA.

From this evidence, Kohn and Harris schematized sulfonamide action as follows: among the syntheses in the cell necessary for growth and multiplication there is a special group X (termed *secondary reactions*) into which enter substances (including methionine and peptone) the production of which is catalyzed by PABA (termed *primary reactions*). When the stores of X fall below a critical concentration, growth rate decreases. PABA always remains effective as an antagonist because the primary reactions now have available PABA and all the secondary reactions which follow are restored. It is the primary reaction involving methionine synthesis which is inhibited by low concentrations of sulfonamides, since this inhibition can be antagonized by PABA as well as methionine. As the drug concentration is increased more primary reactions become inhibited, and the synthesis of the other X components, in turn, is inhibited.

Two objections can be raised against this theory: first, as already stated, the delay in sulfonamide action is not always observed; second, as pointed out by Sevag *et al.* (246), there is no known example where an excess of enzyme produces an inhibition, and it is well-established that PABA acts as an inhibitor in concentrations above those which antagonize sulfonamide activity. PABA antagonism has been observed in sulfonamide-inhibited systems where its action would be difficult to conceive as catalytic (e.g., the carboxylase and charcoal systems already referred to). Before accepting such a complicated explanation for PABA antagonism which, besides having the above objections to it, could apply to only one phase of sulfonamide-antagonism, it seems best to attempt to find a simpler explanation which would apply to all instances of this antagonism.

h. As detoxicant. Among the various capabilities of the relatively simple compound PABA, other than that of sulfonamide-antagonism, is its ability as a detoxicant. Although unable to counteract the trypanocidal action of pentavalent arsenicals in infected mice, it does protect the animals against toxic doses of the drugs (231). A similar protection is afforded mice against the toxicity of the trypanocidal drug Stibosan (sodium *m*-chloro-*p*-acetylaminophenyl sti-

bonate), a pentavalent antimony compound (280). Here again there is no interference with the drug's trypanocidal potency *in vivo*.[19]

Whether or not the action of PABA in these instances is in any way related to PABA-antagonism of sulfonamide action is not yet known. Actually, the phenomenon of antagonism of sulfonamide inhibition of cell growth may be regarded as a detoxication, and it may be that both detoxications are an expression of some fundamental property of the PABA molecule.

Summary: PABA has been isolated from yeast and characterized; it antagonizes sulfonamide inhibition of bacteria *in vitro* and *in vivo*, and of many other cells, on a competitive basis with the sulfonamide; it behaves as a growth factor for several non-pathogenic bacteria; it has not been shown conclusively that it acts as a vitamin in any case; it acts as a detoxicant for certain antimony and arsenic compounds; it is regarded by the Woods-Fildes theory as an essential metabolite which is displaced from its enzyme by sulfonamide, a proposal for which there has never been any direct evidence; there is no consistent relationship between sulfonamide-antagonist production by bacteria and their susceptibility to sulfonamides; PABA antagonizes sulfonamide inhibitions of systems in which it cannot possibly be an essential metabolite; it has also been proposed as a catalyst in the cell, a theory which can be seriously questioned. A critical analysis of these facts will be deferred to the final section, but it may be stated here that the only definite conclusion that can be made at present is that PABA by some type of adsorptive phenomenon counteracts sulfonamide inhibition.

2. Other Sulfonamide-Antagonists of Known Composition

a. Methionine. The action of methionine is very interesting and has received considerable attention. Methionine has been found to antagonize the action *in vitro* of SA, SP, ST, SD and SG, and, with but a few exceptions, under conditions in which no growth-stimulation is observed with the methionine alone (15, 91, 95, 126, 262). The $l(-)$ form is about ten times as effective an antagonist as the $d(+)$ form (91). Strauss *et al.* (262), working with *E. coli*, found that methionine can reverse SG and SA, though it is less active than PABA. No reversal of bacteriostatic concentrations of SP, ST, and SD was observed, thus suggesting that these compounds may act at some loci in the cell other than those affected by SA and SG. Other experimental results have indicated this (91, 128, 263).

Harris and Kohn (91) found that methionine is effective only against low concentrations of sulfonamides, and, as with PABA, higher concentrations are required to exert antagonism on sulfonamides other than SA. On the other hand, observations (15, 91) indicate that unlike the condition existing with PABA, there seems to be no constant ratio between the amount of SA present and the amount of methionine required to neutralize it.

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[19] SA itself is reported to exert a definite protective action against liver necrosis from acute carbon tetrachloride poisoning in rats (71). A sparing effect of SP on the intoxication of the leucopoietic tissue by benzene has been demonstrated in rabbits (168). PABA failed to inhibit the leukotoxic action of benzene.

Bliss and Long (15) reported that methionine concentrations of 1 per cent or more have an inhibitory action on *E. coli*. They also made the interesting observation that SA is able to neutralize this methionine inhibition to a certain extent. It was their experience that the range of SA concentrations over which methionine is capable of antagonism is very narrow, although the range of effective methionine concentrations is very extensive. *The former observation might account for the failure to obtain methionine antagonism of sulfonamide action in some instances.*

The metabolism of methionine in bacteria is almost as obscure as that of PABA. Bacterial growth does not occur if the NH_3 of the basal medium is replaced by methionine (91). It is not oxidized, decarboxylated, deaminated, or hydrolyzed by washed suspensions of *E. coli*. Therefore, the role of methionine in the metabolism of *E. coli* is neither to supply nitrogen nor energy for growth. Kohn and Harris (127), however, recently obtained a culture of *E. coli* requiring methionine as a growth factor by growing the bacilli in an amino acid-purine mixture containing SA. Cultivation in SA alone, or in methionine alone, did not alter the methionine requirement of the organism. The apparent paradox was explained by suggesting that SA-resistance developed in methionine-free medium involves adaptive metabolic pathways which protect methionine synthesis, whereas, in media containing methionine, such adjustments are not necessary.

Although there is no doubt that methionine can antagonize sulfonamide inhibition, there is no conclusive indication as to its mechanism of antagonism. Bliss and Long (15) suggest, without evidence, that methionine, and perhaps arginine and lysine (which show some sulfonamide-antagonistic activity), are not themselves anti-bacteriostatic, but rather are precursors of a substance which has such activity, and is an essential metabolite whose production is hindered by sulfonamide. The explanation offered for methionine antagonism by Kohn and Harris has already been discussed.

b. Amino acids, purines, urethane, etc. Various amino acids and purines have been shown to counteract sulfonamides under certain conditions: arginine and lysine (15); glutamic acid, glutamine and casamino acids[20] (95, 179, 205); aminoids[21] (205); glycine, serine, allothreonine, guanine and xanthine in a medium containing methionine, their individual affects being additive (128).

As stated above, guanine and xanthine in the presence of methionine antagonize SA inhibition, but in the absence of methionine, guanine and xanthine increase SA action, although having no effect on growth in basal medium, with or without methionine (128). Hypoxanthine and adenine potentiate SA inhibition, with or without methionine; in the absence of SA these 6-purines are without effect on growth. Thus, the ability to potentiate methionine's antagonism of SA inhibition apparently is dependent on substitution at positions 2 and 6 of the purine nucleus; potentiation of SA inhibition, on the other hand, seems associated with substitution in position 6 alone. When SA action is completely counteracted by PABA, all these purines are without effect. Further-

[20] *Acid hydrolysate of casein.*
[21] *Trade-name of biuret-free material used in culture media.*

more, bacteria (*E. coli*) made resistant to SA possess a changed response to the purines; growth is inhibited by hypoxanthine and adenine but not by xanthine or guanine; SA addition does not alter this effect, but PABA or methionine abolishes it completely. All of these observations suggest that metabolic relations for SA, methionine and purines exist which are as yet unknown.

Snell and Mitchell (256) found that, although inactive alone, in the presence of suboptimal amounts of PABA, the purines adenine, guanine, xanthine, and hypoxanthine further antagonize sulfonamide action on *Lactobacillus arabinosus* and *L. pentosus;* methionine showed no antagonistic action under similar conditions[22]; with *L. pentosus* and *L. casei*, on the other hand, antagonism is affected by the purines (also by methionine in the case of *L. casei*) without PABA, provided the medium contains biotin concentrate. The nature of the substance present in biotin concentrate which effects purine activity is unknown. These various lactobacilli, as well as others, are stimulated in their growth by these purines and PABA. In contrast to the lactobacilli, the growth of *Acetobacter suboxydans* is not affected by the purines adenine, guanine, and xanthine in the absence of PABA, but these purines act as growth accessories in that they increase the response to PABA, thereby suggesting a relationship between purines and PABA both for growth and for sulfonamide-antagonism (144). These results with adenine and hypoxanthine are conflicting, but it must be emphasized that different bacteria were used in each case. That the difference found is a result of the fact that different bacteria were used, is borne out by evidence obtained by Kohn and Harris (128). Adenine, but not guanine, has been found to annul the chemotherapeutic efficiency of SA in hemolytic streptococcal infection in mice (190).

Glucose has been reported to counteract sulfonamide action, under certain conditions, in concentrations (0.6 to 2.6 per cent) at which the glucose alone does not stimulate bacterial growth (95).

Lamanna and Shapiro (136) found that mercuric chloride can counteract SA bacteriostasis and *vice versa*, neither result being dependent on the growth-stimulating capacity of low concentrations of the antagonist. Mixtures of both substances in stimulatory concentrations proved to be inhibitory. These investigators were of the opinion that such results probably arise because the two substances stimulate or inhibit separate enzymes whose reaction rates are in some way interdependent.

A very important group of substances which has been shown to antagonize sulfonamide action are those primarily related to cell oxidative metabolism, e.g. coenzymes and nicotinic acid. They have commanded considerable recent attention, but since they are more concerned with respiratory functions, they will be considered in the succeeding section.

Urethane, a general cell inhibitor, has been found to antagonize SA inhibition

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[22] In this report it was claimed that methionine failed to exert any additional sulfonamide-antagonistic activity under these specific conditions; however, since the medium employed contained casein hydrolysate it may well be that optimal amounts of methionine were already present.

of growth and luminescence in growing cultures of luminescent bacteria (105). Counteraction of sulfonamide inhibition of the growth of other bacteria has also been observed (173). As with methionine, it is reported that the antagonism by urethane of sulfonamide inhibition is not competitive, the molar ratio: urethane /SA varying approximately between 1 and 100 (173). The mechanism of this antagonism by urethane *in vitro* has recently been explained satisfactorily by Johnson *et al.* (109) on the basis that the sulfonamide and urethane combine reversibly with each other to form an inactive complex. This will be discussed in greater detail at the end of this section. Other substances found by Johnson *et al.* to antagonize SA inhibition of bacterial luminescence include ethyl alcohol, butyl alcohol, chloroform, ether, acetone, glycocoll, arginine, and xanthine.

The substances of known composition which are reported to antagonize sulfonamide inhibition thus include PABA and its derivatives, methionine, certain purines, certain amino acids, glucose, mercuric chloride, coenzymes, nicotinic acid, and urethane.

3. Sulfonamide-Antagonists of Unknown Composition

a. Proteins (serum, etc.) Meat extract and infusion, blood, plasma, serum, various exudates and transudates, albumin, gelatin, casein, fibrin, edestin, and sterile nutrient broth interfere with the activity of sulfonamides, *in vitro*, though to a less extent than peptone (17, 77, 96, 179). The products of protein digestion apparently have greater power of antagonizing sulfonamide than the parent protein (87).

The mechanism of antagonism of these substances is not definitely known. Boroff *et al.* (17) were of the opinion that the antagonistic activity of such material could not be explained purely on the basis of growth-promotion. These various substances have never been directly assayed chemically for PABA; and, pertinent to the question of whether protein antagonism is due to its PABA content, it is highly significant that no aryl amine has ever been demonstrated in protein. It is doubtful whether protein gives the bacterium mechanical protection from sulfonamide, for other substances such as gum, starch, and saponin, which should protect in a similar manner, have no such action (77).

One series of investigations, however, does give a clue to the mechanism whereby protein material antagonizes sulfonamide inhibition. Early cataphoretic studies indicated that Prontosil, but not SA, is adsorbed onto serum albumin and bacterial protein but not to globulin (239, 257). Kimmig and Weselmann (119), also using the cataphoresis technique, confirmed the negative results with globulin, but found all the sulfonamides studied, which included Albucid, Neo-Uliron and SP, to be adsorbed onto serum albumin. That the combination is an adsorption was indicated by the finding that dissociation could be produced by the addition of animal charcoal. By means of ultra-filtration, it was shown that the sulfonamides are held to the proteins by a pressure exceeding eight atmospheres. Davis and Wood (40, 41), using an entirely different experimental approach to the problem, confirmed the latter findings. Their conclusions were based on dialysis experiments which indicated that sulfonamides are ad-

sorbed onto albumin but not globulin. In normal plasma 20 per cent of the SA present is bound, 40 per cent of SP, 55 per cent of SD, and 75 per cent of ST. Experiments with *E. coli* in synthetic medium, with and without albumin added, suggested that the concentration of unbound sulfonamide determines the level of bacteriostatic activity, the bound sulfonamide being apparently inactive (40). The data indicated that it is the anion of the sulfonamide which becomes bound, and, if true, the binding tendency should be proportional to the dissociation constant and to the bacteriostatic activity of the sulfonamide. McClintock and Goodale (169) placed SA in albumin solutions undergoing digestion with trypsin and demonstrated that a combination takes place between the ring amino group of the SA and the albumin, and also the early hydrolytic products of the albumin. This conjugate was bacteriostatically inactive.

Sevag *et al.* (245, 246, 250), after demonstrating antagonism by peptone, serum albumin, and globin of sulfonamide inhibition of bacterial growth and respiration, of catalase activity, and of carboxylase activity, suggest that antagonism in such instances may be due to the ability of these substances to favor the dissociation of the sulfonamide from the inhibited enzyme's surface by the formation of an inactive sulfonamide-protein complex, i.e., the peptone or protein has greater attracting or adsorptive power for the sulfonamide than the enzyme or enzymes which the sulfonamide would inhibit in their absence (cf. 128). Johnson *et al.* (109), in their recent experiments on antagonism of SA inhibition of bacterial luminescence, obtained evidence that serum and peptone owe at least part of their antagonistic activity to their SA-combining power. Whether or not formation of an inactive complex can completely account for the antagonistic activity of these various materials cannot at present be answered; it seems fairly certain, however, that it is at least part of the story.

b. Peptone. The first material discovered to have sulfonamide-antagonistic properties was peptone (15, 91, 157, 177, 205).[23] Lockwood and Lynch (159) showed that this phenomenon is general, peptone being antagonistic to SA, SP, and ST in the case of pneumococci, staphylococci, colon bacilli and hemolytic streptococci. According to Lockwood (157), bacteria under the influence of sulfonamide are unable to break down complex protein but are still able to utilize for growth simple protein-split products, such as peptone.

Lockwood's proposal has been tested by various methods and found to be untenable. Long and Bliss (160) tested a known proteolytic streptococcus to see whether it was more or less affected by SA in peptone-free horse and rabbit sera than a non-proteolytic strain. The two strains grew equally well in the presence or absence of SA in broth. In serum, the proteolytic strain grew more rapidly but the difference between the growths of the two strains was not altered in any way by SA. Furthermore, a direct estimation of the activity of streptococcal proteolytic enzyme has revealed no change in activity under the action of SA (77). Abderhalden (1) found that the sulfonamide derivatives Uliron,

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[23] No distinction will be made between peptone, neopeptone, or proteose-peptone though in some instances there may be a difference in their action, qualitatively (160) as well as quantitatively (153).

Albucid, SP, ST, and Prontosil, in concentrations of 20 and 200 mg% have no influence on the action, *in vitro*, of pepsin, trypsin, and serum di- and polypeptidases.

After the discovery of the great sulfonamide-antagonistic powers of PABA it was perhaps natural to think that the counteracting power of peptone (and other antagonists of organic origin) may be due to its PABA content, and claims to this effect were made (174). These claims were based on non-specific chemical assays or on microbiological assays.

Absence of PABA in Peptone. There have been several indirect indications that peptone's antagonistic activity cannot be due to its PABA content (91, 126), but the final word in such a question depends on whether or not PABA can be positively detected in peptone. Eckert (49), using a modified Marshall method for the determination of PABA, found that peptone broths sometimes gave a slight color reaction which he ascribed to the presence of tryptophan. Kohn and Harris (128), following Blanchard's (14) technique and the analysis of Bratton and Marshall, found the PABA content (bound and unbound) of various peptones to be very small. Evidence was obtained by Kohn and Harris which led to the following interpretation of peptone antagonism: The active substances in peptones can be divided into two groups, the first composed of the four amino acids methionine, glycine, serine and allothreonine, and the second made up of the purines xanthine and guanine, which are only antagonistic to sulfonamide action when in the presence of methionine and which potentiate sulfonamide action in its absence. The purines do not affect the competition between PABA and the sulfonamides, this being interpreted by Kohn and Harris to mean that purines are secondary antagonists and potentiators. Possibly the small amount of PABA found in peptone plays a part. The summation of all these factors accounts for almost all the antagonism against SA. Against the heterocyclic sulfonamides, however, this group accounts for nearly all the antagonism only when growth-inhibition is less than approximately 65 per cent. Above this, another factor of great power, whose nature is unknown, is active. It is water-soluble, not a known, naturally occurring amino acid or PABA. It was obtained from pancreas, which seems to be the best source, but is neither insulin nor other protein. The fact that this substance is concerned only with antagonism of the heterocyclic derivatives of SA suggests that these derivatives either act at more than one locus in the cell, or form an inactive complex with some component of peptone and pancreas. The methods used in this investigation ruled out any antagonism due to growth-stimulation.

Peptone is both a growth-stimulator and sulfonamide-antagonist and, as already discussed, it has been suggested that the sulfonamide-antagonism is a result of the growth-stimulation. Further evidence against the "growth-stimulation" hypothesis, appears in the work of Sevag *et al.* (246) who found that peptone counteracts the SA inhibition of respiration when there is no growth. When peptone is added to a culture, growth and respiration increase in parallel with each other. It was observed that the greater the increase, the greater the inhibition by sulfonamide. If sulfonamide-antagonistic action were due to non-

specific growth-stimulation, the opposite would be expected. The experimental findings are in agreement with the idea that to a great extent the respiratory increase is coupled with growth, and that this part of the respiration is sulfonamide-sensitive; thus, it would be expected that the greater the increase in respiration (and growth) the more there is to be inhibited. As already stated, counteraction of sulfonamide inhibition of carboxylase activity in *E. coli* and *S. aureus* by peptone suggested (245, 246) that peptone and bacterial enzyme protein compete for the inhibitor sulfonamide, and as a result of this competition, sulfonamide action is counteracted. It is known that peptone forms reversible complexes with various substances. For example, tannic acid combines reversibly with invertase, precipitating it and thus inhibiting its activity (213); and this inhibition is counteracted by egg albumin or peptone which displace the tannic acid by combining with it. Analogously, protamine and the α- and β-globulins from human plasma inhibit bacteria, their inhibition being counteracted completely by human plasma (244). Since human plasma *per se* is not inhibitory, and in fact is capable of counteracting anti-bacterial substances, it is evident that the α- and β-globulins are neutralized in the plasma by proteins.

c. Necrotic tissues and abscesses. It was an early, clinically important, observation that in the presence of abscesses and tissue necrosis the sulfonamides are helpless (7, 120, 158). Attempts to find sulfonamide-antagonist in pus have been conflicting: MacLeod (179) claimed invariable success, and Fox (74) reported persistent failure. There is, however, a wealth of indisputed clinical and experimental evidence that tissue breakdown products contain sulfonamide-antagonists. It is apparent, for example, that whether or not sulfonamides can produce bacteriostasis in the presence of these products depends on the concentration of sulfonamide which can be obtained in that area. Sulfonamides have given good results in pyogenic infections where they can be applied locally in high concentration. On the other hand, the presence of large amounts of sulfonamide-antagonist in tissue breakdown products, as compared to the relatively small amounts in serum, explains the marked difference in therapeutic response between rapidly spreading infections, such as erysipelas or pneumonia, and localized purulent foci that can only be reached by the ordinary therapeutic blood levels which are too low to cope with the antagonists present in the purulent foci. Dead bacterial cells also have a sulfonamide-antagonistic effect (157).

It is not known whereby, or even what, substances present in tissue necrosis and abscesses antagonize sulfonamide-action; certainly PABA has not yet been identified in such loci. In fact, it seems improbable that the antagonistic activity in these loci is due to PABA. The sulfonamide-antagonistic action of PABA is limited to a zone of concentration with definite limits (D1d), and if the failure of sulfonamides in the presence of tissue necrosis were due to PABA, it would seem to be somewhat of a coincidence that, in every instance, the PABA concentration should lie within the particular concentration range capable of antagonizing the concentration of sulfonamide drug present. Such a relationship would not be required if a substance such as peptone were responsible for the sulfonamide-antagonism, for peptone concentration can be greatly increased above its antagonistic level without disappearance of the antagonism (D3b).

d. Tissue Extracts. Sulfonamide-antagonist has been found in various normal animal tissues (including muscle, liver, kidney, pancreas, and spleen), turnip, enzymatic casein hydrolysate (not acid or alkaline hydrolysate), transplanted rat sarcomas, and some higher fungi (including the common mushroom) (83, 179, 284). The antagonists obtained from these sources are to a greater or lesser degree in a conjugated form. Some of the properties of these naturally occurring antagonists (e.g., ether solubility) are similar to those of PABA

but some of the antagonists have been found to differ chemically in several respects from PABA (179).

Various body fluids and tissue extracts have been analyzed for PABA content by Lewis (153) and Landy and Dicken (140) by microbiological assay methods. All body fluids and extracts (urine, blood, liver, etc.) thus tested were found to contain PABA, appreciable amounts of which in many instances were in an inactive form, being activated by alkaline hydrolysis. Using the variant of *Neurospora crassa* unable to synthesize PABA (section D1a) in a biological assay for PABA, Bonner (16) found tomato roots to contain PABA or a substance with similar action. Using the same assay method, Mitchell *et al.* (200) assayed beef liver, spinach, oat seeds, mushrooms and fresh yeast for PABA and concluded from their results that the assay procedure of Landy and Dicken responds to only a fraction of the total amount of PABA obtainable after acid or alkaline hydrolysis. It was also found that enzymatic hydrolysis or autolysis is not always sufficient to lead to the maximum effect. Separation of partially purified liver fractions by a "chromatogen technique" has also indicated the possible presence of PABA in this tissue (29).

Using a modified Marshall method for detection of PABA, Eckert (49) was unable to obtain a positive reaction with blood filtrates from normal animals. Kisch and Strauss (122), on the other hand, report that normal blood and urine contain small amounts of chromogenic material which upon diazotization gives the color reaction of PABA. Teply *et al.* (269) found a counteracting acid-labile factor(s) present in liver extracts and grass juice which was distinct chemically and physically from PABA and which had properties similar to those reported for folic acid preparations. It is seen, therefore, that although sulfonamide-antagonists undeniably exist in various tissues, and although some of these may be PABA, this compound has not unquestionably been identified with any of them.

e. Bacterial products. Certain bacteria have been shown to give off or contain sulfonamide-antagonist (179); in a few instances, failure to find antagonist in bacteria has been reported (179). There seems to be no doubt, however, that antagonists are produced by certain bacteria at least. The question of most importance is their identity, and it has already been dealt with in connection with the discussions relative to sulfonamide-fastness (D1f). One important investigation which was not mentioned, however, is that of Landy *et al.* (141). Culture filtrates and hydrolyzed whole cultures were assayed for PABA by the method of Landy and Dicken (140) which depends on the specificity of the need of *Acetobacter suboxydans* for PABA as a growth factor. Using this criterion, all organisms studied elaborated PABA to a greater or lesser degree. The organisms included strains of the genera *Staphylococcus*, *Streptococcus*, *Bacillus*, *Brucella*, *Corynebacterium*, *Eberthella*, *Escherichia*, *Clostridium*, *Klebsiella*, *Mycobacterium Proteus*, *Salmonella*, and *Shigella*.

As to whether these sulfonamide-antagonistic bacterial products are PABA it can only be repeated that, although some of them may be PABA, so far only tests have been used, the specificity of which can be questioned. It has been demonstrated rather definitely that at least some of these antagonists act by a non-specific growth-stimulation (82, 83).

Summary: A great number of substances with a great diversity of source antagonize sulfonamide inhibition. The first question to be answered is whether their antagonistic activity is due to their PABA content. This obviously cannot be the case with methionine, urethane, glucose, and other substances of known composition. For the remainder of the antagonists, those of unknown composition, it was thought, after the appearance of the Woods-Fildes theory, that they owe their activity to their PABA content. This assumption still persists largely today. The assays which have been employed to determine the presence of PABA in these substances fall into three groups as follows: First is the "sulfonamide-antagonistic" assay. This is obviously non-specific;

no more proof of this is needed than to look at methionine and urethane. Second, physical and chemical properties such as ether-solubility and diazotizability. Solubility is obviously non-specific and diazotizability merely indicates a primary arylamine. The danger of assuming such diazotizable substances to be PABA has been emphasized by reports that bacteria can produce diazotizable aromatic amines which apparently are not PABA. Miller (197) found that filtrates of both susceptible and resistant streptococcal cultures, although containing a primary aromatic amine as shown by diazotization and coupling with dimethyl-α-naphthylamine, had no antagonistic effect on sulfonamide action. Fox (73) claimed to have found a diazotizable aromatic amine formed by bacteria during sulfonamide bacteriostasis; but it was not PABA, its formation being prevented by PABA antagonism of the sulfonamide action. In the third group fall the microbiological tests. Although Landy and Dicken (140) showed that of fourteen related compounds none possessed more than one-tenth the growth-factor activity of PABA for *Acetobacter suboxydans*, one can still question the specificity of the test. Mirick (199) has recently pointed out several possible sources of error in such bioassays. Furthermore, there is evidence that sulfonamide-antagonism and growth-factor activity cannot be assumed to be associated phenomena (D1d). Thus, there is no absolutely conclusive proof that PABA is present in these substances. This is of especial significance in the case of bacterial products, because demonstration of PABA production by bacteria is vital to the Woods-Fildes theory. Chemical or physical analysis of a universally recognized specific nature must be performed on all these various substances to decide the issue. So far, such analysis has been applied to yeast, in which PABA was found, and to peptone, in which PABA was found only in insignificant quantities.

As to how sulfonamide-antagonists act, sufficient data are at hand to warrant the conclusion that interference with sulfonamide action can be divided into at least three distinct categories. In the first, the antagonism is by some specific interference with sulfonamide action. PABA certainly is an example of this type of antagonist. Methionine and mercuric chloride also appear to be representatives of this group. The observation that amino acids containing aromatic groups (tryptophan, tyrosine, and phenylalanine) inhibit SP adsorption onto activated carbon particles suggests that part of the antagonistic activity of amino acids may be due to a similar action at the loci in the cell where sulfonamide adsorbs (146). Antagonism by specific interference will be discussed further in the last section. In the second category, antagonism is unspecific by growth-stimulation, i.e., growth-stimulation by an action on the cell unrelated to the mechanism of sulfonamide inhibition. A prerequisite for this phenomenon is sub-optimal growth to begin with. It is known that sulfonamide action is greater the poorer the nutritional environment of the organisms (77, 83, 193). On the other hand, it must be emphasized that the mere possession of growth-stimulatory properties does not mean that a substance will antagonize sulfonamide action (91, 160, 165, 181, 205). In fact, one compound, asparagine, has been found to enhance sulfonamide activity in spite of the fact that in the

absence of sulfonamide it is a growth-stimulant (233). There is no doubt that the two phenomena, growth-stimulation and specific interference, are not associated. This was first shown by Lynch and Lockwood (165) who demonstrated that though some substances such as peptone, act both as growth-stimulants and as a sulfonamide-antagonists, there are other substances which exert either growth-stimulation or sulfonamide-antagonism alone, PABA being a representative of the latter group. That PABA does not stimulate growth (except when a growth factor) in concentrations which counteract sulfonamide action (or in any concentration for that matter) has been adequately confirmed (136, 196, 248). Further evidence for this dissociation appeared when it was found (91, 126) that, out of an extensive list of amino acids tried, only methionine and PABA exerted sulfonamide-counteracting action, although many of them stimulated growth more than methionine in the absence of sulfonamide. As has been seen, PABA does not belong to this group, nor can any considerable part of the activity of methionine or peptone be considered as belonging here. Certain nutrient substances such as glucose and certain amino acids, however, undoubtedly owe at least part of their antagonistic activity to a non-specific growth-stimulatory action. In the third group, antagonism results from the formation of an inactive complex between sulfonamide and antagonist. That such direct interaction may account for PABA-antagonism of sulfonamide action has received but little consideration until recently, because of the very large molar ratios of PABA/SA observed in antagonism; and it was argued that the molar concentration of PABA required for antagonism should be of the same order as that of the sulfonamide if antagonism were a result of an interaction between the sulfonamide and PABA. But as seen in section B3d, the ratio of "active" sulfonamide to PABA is near unity. Direct procedures have never been used to determine if PABA does combine with sulfonamides, but the recently reported results of Johnson et al. (109) are fairly conclusive on this point. These investigators showed that when SA and PABA inhibit bacterial luminescence they do so with certain definite group characteristics. When both inhibitors are present simultaneously, there is no combination between PABA and SA. In contrast to such combinations of inhibitors in which both inhibitors are of the same type (space does not permit a description of the characteristics of each type inhibitor according to Johnson et al.), combinations of different type inhibitors can result in antagonism or synergism, depending on temperature and concentration conditions. Such instances of antagonism are a result of the formation of an inactive complex by a reversible adsorption, perhaps through hydrogen bonds. This apparently is the mechanism of sulfonamide-antagonism by urethane, and also probably in part of the antagonism by various other substances. Adsorption between protein and sulfonamide has been adequately demonstrated by various techniques, and it has been suggested that this, to a major extent at least, is the mechanism of antagonism by proteins and protein degradation products, such as peptone.

There is, therefore, antagonism by specific interference, by non-specific growth-stimulation, and by inactive complex formation. Henceforth it should

be useful to ascertain in which of these categories a given antagonist belongs. Two things, however, must be emphasized: first, one antagonist might fall into two or even all three categories, and second, other mechanisms of antagonism are conceivable and must not be lost sight of. The various possible ways in which an antagonist could antagonize inhibitor action are reviewed in the last section.

E. EFFECTS ON RESPIRATORY MECHANISMS

The fundamental action of sulfonamides is inhibition of cell multiplication. This is an *effect* of sulfonamide action, and preceding sections have dealt with observations made on this effect and resultant theories as to its *cause*. We now proceed to a consideration of phenomena believed to be more closely related to the cause. One of the functions of all cells is respiration, and this can be measured with relative ease and accuracy. This section deals with the observations and their interpretations of the effects of sulfonamides on the respiratory mechanisms of bacteria and other cells.

1. General Considerations

It is common knowledge that the energy for cell division and growth, as well as for maintenance, and for synthesis of any essential metabolite, etc., must come from respiratory oxidative processes. These oxidative-reductive respiratory processes are catalyzed by the dehydrogenases, the flavoproteins, the cytochromes, cytochrome oxidase, etc. If any inhibitor blocks the activity of any one enzyme in a respiratory chain the overall activity of the chain is decreased to the same extent, provided, of course, that there is no shunt or by-pass available through which the uninhibited components of the chain can continue to function. Furthermore, if these energy providing respiratory reactions are inhibited, cell division and growth are also inhibited. Sulfonamides could conceivably play the part of a respiratory enzyme inhibitor and thereby bring about their bacteriostatic effect. Several investigators have tested this hypothesis, and several have made experiments to determine which step if any in the chain of respiratory enzymes is inhibited by sulfonamides. Any theory of sulfonamide action based on inhibition of respiratory processes hinges on whether or not an inhibition of these processes can be observed experimentally, e.g., a decrease in aerobic oxygen consumption or anaerobic CO_2 production during sulfonamide action.

2. Inhibition of Bacterial Respiration

Barron and Jacobs (10) demonstrated a slight inhibiting effect by low concentrations (0.01 M; 173 mg %)[24] of SA on the oxygen consumption of heavy

[24] It will be noted that this concentration as well as many others quoted in this section are very high as compared to the concentrations usually used in such experiments as referred to in previous sections (in the range of a few to about 50 mg%). The reader will recall that an inverse relationship exists between inoculum size and concentration of sulfonamide required for inhibition (section B3b); these high concentrations are therefore

saline suspensions of resting streptococci and Friedländer's bacilli in the Warburg apparatus, but no effect on *E. coli* and gonococci. Chu and Hastings (31), using higher concentrations of SA (ca. 0.04 M; 692 mg %), found inhibition of respiration in several experiments with suspensions of washed pneumococci; similarly resting cultures of gonococci and meningococci, although poor respirers, were also inhibited; in the case of *Streptococcus pyogenes* the respiration was so small that experimentation proved to be unsatisfactory. The inhibitions of respiration reported by these two groups of workers fell into the range of 5 to 50 per cent.

Respiratory inhibition of resting and actively dividing dysentery bacilli by SA, ST and SP has been demonstrated by Dorfman and coworkers (46, 47). Ely (50) obtained approximately 50 per cent inhibition of oxygen consumption of resting *E. coli* in synthetic medium and in rabbit serum by 0.06 M (1000 mg %) and 0.05 M (830 mg %), respectively. Kohn and Harris (126) also reported that the oxygen consumption of suspensions of *E. coli* in phosphate buffer + $MgSO_4$ + $NaCl$ was not affected. In saline-glucose medium, at lower sulfonamide concentrations, it was claimed that the oxygen consumption per bacterium did not fall appreciably, and in higher concentrations there was a decrease in oxygen consumption, but it lagged behind the fall in growth rate. The authors concluded that sulfonamides do not have a direct influence on respiratory enzyme action; but calculations made from their published data show that there was inhibition of respiration in all but one instance (see table 3).[25] Unfortunately, a correlation between inhibition of respiration and inhibition of growth cannot be made, because growth rates were determined from the rate of oxygen consumption rather than from direct bacterial counts.

3. Correlation of Respiratory Inhibition with Growth-Inhibition

As can be seen from the reports cited above, it was not possible on the basis of these studies to learn whether or not the inhibition of growth by sulfonamides was the result of the inhibition of the bacterial respiratory enzymes. A more complete set of experiments carried out by Sevag and Shelburne (247, 248) to determine the effect of SA on respiration and growth of *S. pyogenes* and pneumococcus Type 1 showed a definite relationship between these two effects. After measuring simultaneously at various time intervals the increase in the number

considered low in view of the large inocula used in such experiments. Justification for using such large inocula and concentrations will be given in part 7 of this section.

[25] It has been known for some time (215) that *E. coli* produces hydrogen gas, which of course renders unreliable experiments based on manometric measurement of oxygen absorbed or carbon dioxide produced unless this is taken into consideration and accounted for. Sevag and Jane Henry (246) have recently observed that this production of hydrogen from glucose by *E. coli* is inhibited by sulfonamides (0.04 M; 690 mg % SA) and that this inhibition does not necessarily parallel the inhibition of oxygen consumption. The above-mentioned reports apparently overlook this phenomenon of hydrogen production; accordingly all such experiments with *E. coli* will have to be reinvestigated. At the higher inhibitions obtained, however, there is little doubt that oxygen consumption was being inhibited.

of streptococci (and the mg of streptococcal nitrogen) and respiration in the presence and absence of SA (0.04 M; 690 mg %), they concluded that the

TABLE 3

Calculation of inhibition of respiration per cell of E. coli in a medium supporting growth; data for calculations obtained from Table 2 of Kohn and Harris (126)

SULFONAMIDE CONCENTRATION		CMM. O_2 CONSUMED PER HOUR PER 10^7 CELLS (FROM PLATE COUNT)	% INHIBITION OF O_2 CONSUMPTION PER CELL
	0	2.7	
	10^{-4} M	2.6	4
	10^{-3} M	1.7	37
SA	0	2.2	
	10^{-4} M	1.9	14
	10^{-3} M	1.4	36
	0	2.3	
	3×10^{-6} M	2.2	4
	3×10^{-5} M	1.0	57
SP	0	1.9	
	3×10^{-6} M	1.8	5
	3×10^{-5} M	1.1	42
	0	2.5	
	10^{-6} M	2.4	4
	10^{-5} M	1.7	32
ST	0	1.8	
	10^{-6} M	1.9	*
	10^{-5} M	1.7	6

* Apparently there was stimulation rather than inhibition in this instance.

inhibition of both the aerobic and the anaerobic respiration results in proportional inhibition of growth. After recalculation (figure 1) of the data (for *S. pyogenes*) reported by these investigators on a per cell or mg N basis[26] it is apparent that

[26] In certain instances, respiratory data have not been reported on a per cell basis. No conclusions concerning the presence or absence of respiratory inhibition in the individual cells can be drawn unless this is done, for a change during the experiment in the number of cells present must affect the base line from which the inhibition is judged. As a matter of fact, in certain instances, data expressed even on a per cell basis may be misleading as a result of changes in cell size during the course of the experiment or of different cell sizes under different experimental conditions. In such instances the data should be expressed on a mg N (Q_{O_2}) basis (78). A method of measuring growth in bacterial suspensions which is gaining in popularity, namely the photometric method, does not allow for variations in cell size or in non-parallelism between turbidity and viable cell account. These factors should always be checked. It is mandatory that the investigator be certain that his method of growth measurement is providing values sufficiently accurate for the interpretations based on them.

Several other works (90, 234), in reporting experiments of sulfonamide action on growth and respiration of bacteria, gave no indication of whether the inhibition of respiration as reported was on a per cell (or Q_{O_2}, etc.) basis; in some instances, data which the reader

in actively growing cultures, sulfonamides produce a definite inhibition of respiration coincident with inhibition of cell multiplication. Approximately 65 per cent inhibition of aerobic respiration or approximately 45 per cent inhibition of anaerobic respiration results in (or accompanies) complete bacteriostasis. The inhibition of both aerobic and anaerobic growth paralleled the inhibition of respiration. Low concentrations of PABA were capable of completely counteracting the growth-inhibition by SA. It was shown (247) that whether or not the cultures exhibited active growth depended on the nature of the
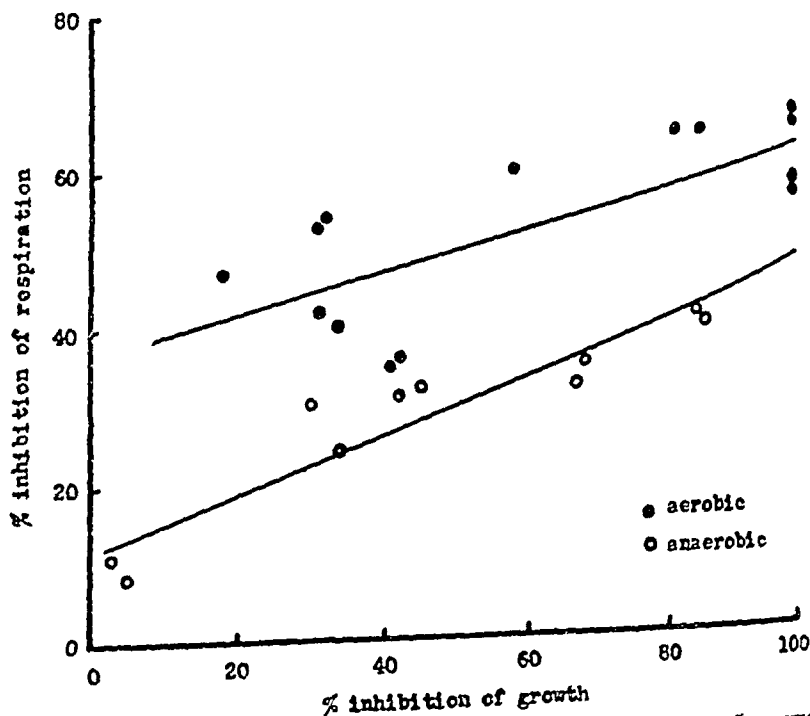

FIG. 1. CORRELATION OF INHIBITION OF AEROBIC AND ANAEROBIC GROWTH WITH INHIBITION OF AEROBIC AND ANAEROBIC RESPIRATION OF STREPTOCOCCUS PYOGENES; CALCU-LATED FROM DATA IN TABLES 3 AND 4 OF SEVAG AND SHELBURNE (247)

medium. Suboptimal media permitted fairly active respiration of *S. pyogenes* but no measurable growth. The respiration of the bacteria in such a state was

would require to determine this for himself were either never obtained or not published. Apparently these workers were going on the assumption that the oxygen consumption per cell would not be decreased, and were using oxygen consumption measurements as an indirect method of determining growth rate—a method which has been used in following normal bacterial growth and which has been proposed as a method of evaluation of germicides (23). It is suggested that, inasmuch as several have reported definite inhibition of bacterial cell respiration, such an assumption cannot be made.

SA-sensitive, but less so than that of bacteria in active growth phase. The fact that the added respiration concomitant with growth is relatively more sulfonamide-sensitive indicates strongly that it is intimately associated with bacterial growth and multiplication.[27]

Clifton and Loewinger (32) claimed to have found that SA markedly inhibits the oxygen consumption of washed suspensions of *E. coli* in the presence of various substrates (these investigators were aware of the fact that *E. coli* produces hydrogen). However, in this publication the calculations of respiratory inhibition were not made on a per cell (or similar unit) basis, nor were data given permitting such a calculation for aerobic respiration. Thus a conclusion with regard to the existence of an inhibition of oxygen consumption is not permitted here. Sufficient data were, however, given for an analysis on this basis under anaerobic conditions. The analysis shows that SA (0.00063 M; 11 mg %) inhibits CO_2 production coincident with inhibition of division, both inhibitions being antagonized by PABA.

Thus it is seen that there is good evidence that sulfonamide inhibition of bacterial multiplication is directly related to the inhibition of respiratory mechanisms, either aerobic or anaerobic.

4. Relation Between the Structure of Sulfonamides and Coenzymes

Various authors have drawn attention to the similarity between the structures of various sulfonamides and coenzymes of respiratory systems (60).[28] Moreover, there is a considerable body of evidence, to be considered below, indicating some relation between sulfonamide action and the normal function of coenzymes.

With these as a background, Sevag and coworkers (248, 251) enunciated explicitly the idea that the chemotherapeutic substances which have structural similarity to the whole or part of the coenzyme molecules may combine specifically with the specific proteins (of the dehydrogenase-containing coenzyme I or II, or of flavoproteins functioning as dehydrogenases, or of other enzymes such as carboxylase, etc.) of the respiratory enzymes. This might result in displacement of the coenzyme by the drug or the formation of an inactive "drug-protein-coenzyme" complex. In such cases, as where drugs are active but do not possess structural similarity with the known coenzymes, mutual affinity

[27] A similar phenomenon has also been observed in the SA-inhibition of bacterial luminescence, i.e., the inhibition is generally less when the metabolism is low (109).

Sevag and Shelburne (247) also showed that HOSA, *p*-aminobenzenesulfonhydroxamide, benzenesulfonhydroxamide, benzhydroxamic acid and hydroxylamine inhibit both the aerobic and anaerobic respiration of hemolytic streptococci. Sevag *et al.* (250) demonstrated that HOSA (also hydroxylamine) is capable of inhibiting non-heme type of enzyme systems as well as the heme type. It must be remembered, however, that there is evidence (section C) that oxidized sulfonamides exert their action by a mechanism differing, in at least some respects, from that of the reduced sulfonamides.

[28] For the role of coenzymes, nicotinic acid, thiamin, etc. in bacterial metabolism see Stephenson (261). Dickens (42) called attention to the similarity of the inhibitors pyridine, quinoline and acridine compounds to the active pyridine group of coenzymes and suggested that they may displace the coenzymes by preferential adsorption on the protein carrier, forming an inactive complex.

between the drug and the specific enzyme proteins was considered as a definite possibility. Such an affinity is certainly not without precedent, as will be referred to presently.

Accordingly, SP would be a potential competitor of coenzymes I and II (di- and tri-phosphopyridine nucleotides: DPN and TPN), and ST (likewise SD) of cocarboxylase. These coenzymes are present in the respiratory systems of most living cells.

Experiments having to do with the inhibition of respiration by sulfonamides have been reported by Dorfman and coworkers (46, 47). Here the inhibition of respiration in the presence of nicotinic acid and its derivatives was studied, dysentery bacilli being the organisms used. They concluded that there is strong indication that SP and SD action is related to the metabolic role of nicotinamide (the pyridine component of the coenzymes). The addition of SP (0.0012 M, 30 mg %) after incubating dysentery bacilli with nicotinamide resulted in respiratory inhibition varying from 8 to 15 per cent. When, however, SP was added before nicotinamide, inhibition of 80 to 95 per cent was obtained. Respiration stimulated by a certain preparation of DPN was inhibited by SP in a manner qualitatively similar to the inhibition of the respiration stimulated by nicotinamide. Much the same results were obtained with ST, the similarity in activity being regarded as not surprising in view of reports of isosterism of the pyridine and thiazole rings (127). ST was also found to inhibit anaerobically the nicotinamide-stimulated fermentation, a result to be expected if ST inhibits reactions involving the nicotinamide-containing coenzymes. SA inhibited aerobic respiration approximately 15 per cent but had no effect on nicotinamide-stimulated respiration; thus, there was no evidence that the small inhibition by SA is related to the nicotinamide-containing coenzymes.

Growth-inhibitions (46) were found to parallel the respiratory inhibitions, with one peculiar exception: PABA completely counteracted ST inhibition of growth but exerted no antagonistic action on ST inhibition of nicotinamide-stimulated respiration. Thus the inhibition of nicotinamide-stimulated respiration seems to be independent of PABA, although it was also discovered that the amount of PABA required to counteract the inhibition produced by a given amount of ST is inversely proportional to the nicotinamide concentration present. Dorfman and coworkers reasoned that if the thesis is correct, that the inhibition of nicotinamide-stimulated respiration is dependent on the ring linked to the sulfonamide group rather than on the SA part of the molecule, then acetylation of SP should not abolish the respiratory inhibition although it should markedly decrease the bacteriostatic activity of the compound. This was found to be the case. Thus, here again is evidence that SP and ST act differently than SA, possibly by acting at metabolic loci in addition to those affected by SA. The interpretation was made that SP and ST, but not SA, compete with nicotinamide or related compounds for enzymes essential for the oxidation of lactate and glucose (or some intermediate derived therefrom).

The respiratory inhibition effected by SP and ST was found to be competitive ince it was dependent on the relative concentrations of sulfonamide and respira-

tory stimulator. Furthermore, the inhibition was reversible since the respiration and growth could be restored by washing the drug out of the cell; this, of course, is evidence for the theory that the sulfonamides inhibit the respiration by forming a dissociable enzyme complex.

These results are not incompatible with those of Sevag and coworkers and Clifton and Loewinger, although it is somewhat difficult at present to bring them entirely into alignment, the approach to the problem in the two instances being different. In the experiments of Dorfman et al. it was difficult to determine the relative importance of respiratory inhibition in stopping growth, although it was shown that nicotinamide (or DPN) can partially antagonize growth-inhibition at certain concentrations of SP or ST.

5. Inhibition of the Dehydrogenases

The dehydrogenases are oxidative-reductive enzymes composed of a protein component and a coenzyme (DPN or TPN). They function by "dehydrogenating" (and thereby oxidizing) substrates. Under normal conditions the reduced dehydrogenase is then oxidized by the oxidative-reductive enzyme next in line, which is in turn oxidized by the next enzyme in line, and so on. In the presence of oxygen, the ultimate hydrogen acceptor in this chain is oxygen. Methods are available whereby a direct measurement of dehydrogenase activity can be made. These consist of adding, in the absence of oxygen, a dye (e.g., methylene blue) which can be reduced by reduced dehydrogenase, and which changes color on reduction. By following the color change the rate of dye reduction can be determined.

Several studies have been reported in which bacterial dehydrogenase activity and the effect of sulfonamides thereon have been measured. MacLeod (178) determined dehydrogenase activity in pneumococci by methylene blue reduction in the presence of various substrates, and found that, whereas the glucose dehydrogenase was not affected by SP in a concentration of 0.0005 M (12.5 mg %), the dehydrogenase activity for glycerol, lactate, and pyruvate was suppressed. Sevag et al. (246), on the other hand, have repeatedly found that both the aerobic oxygen consumption and the anaerobic fermentation of S. pyogenes, Staphylococcus aureus, pneumococcus, and E. coli in glucose are strongly inhibited by sulfonamide. Dorfman and Koser (46) also obtained inhibition of the aerobic and anaerobic metabolism of glucose with SP and ST (but not SA) in the dysentery bacillus. Clifton and Loewinger (32) have confirmed both these observations in E. coli with glucose as substrate and SA as inhibitor, namely, inhibition of aerobic and anaerobic respiration but no appreciable effect on the dehydrogenase activity using methylene blue as hydrogen acceptor. This was interpreted to indicate that the inhibition must occur after the initial activation of the substrate, presumably on a hydrogen-carrier system somewhere between the original dehydrogenase system and the final hydrogen-acceptor. This may be dependent on the relative affinities of SA and methylene blue for the susceptible enzyme.

SA in a concentration of 0.002 M (33 mg %) does not inhibit the dehydrogenation of d-alanine by d-amino acid dehydrogenase to pyruvic acid (21).

Fox (72) has determined manometrically the effects of sulfonamides on isolated enzyme systems of *E. coli*. It was found that, aerobically, the oxidation of lactate alone is markedly inhibited, the oxidation of glucose, succinate, fumarate, malate, and pyruvate being unaffected. Under anaerobic conditions, only pyruvate dismutation is depressed; lactic acid dehydrogenase and glucose fermentation are not inhibited. This sulfonamide inhibition of the lactate system aerobically and the pyruvate-lactate system anaerobically was found to be approximately proportional to the bacteriostatic action in growing cultures with equivalent sulfonamide concentrations. In view of the fact previously mentioned, that *E. coli* produces hydrogen, these experiments by Fox would have to be repeated if this was not taken into consideration (no experimental details were given in the brief report). Bucca (25), working with gonococci, has recently reported that SA inhibits lactic acid dehydrogenase but not glyceric acid dehydrogenase. It must also be emphasized that in experiments such as referred to above in which the Thunberg technique of methylene blue reduction is employed to measure the rate of dehydrogenase activity, if a large inoculum is used in the test a correspondingly large amount of sulfonamide must be used before any inhibition is to be expected, because of the relationship existing between inoculum size and amount of sulfonamide required to produce a certain inhibition of overall respiration and bacterial multiplication (section E7). In general, these results support the theory that during sulfonamide inhibition certain respiratory enzymes are being inhibited.

6. Inhibition of the Respiratory Enzyme Carboxylase[29]

Apropos the suggestion that the sulfonamides interfere with cellular components with similar structure, Sevag *et al.* (251) found that ST (0.0014 M; 35 mg % and 0.0055 M; 137 mg %) and other derivatives containing the thiazole ring exercised decidedly greater specific inhibiting effect on the carboxylases of *S. aureus* and *E. coli* than SP, SD, or SA. It is interesting to note that the specific inhibitory effect of ST on the carboxylases of *S. aureus* and *E. coli* in comparison to SA, SP, and SD is in accord with experimental results, *in vivo* and *in vitro*, of other investigators. Rammelkamp and Jewell (219) found that ST, whether added directly to the blood or administered orally, was superior to other sulfonamides in increasing the blood's bactericidal action against *S. aureus*. Similarly, ST was found superior against growth *in vitro* of *S. aureus* and *E. coli* (262). But it must be emphasized that there is no *a priori* reason why the sulfonamides should give the same results at the same concentrations, even if they all acted by an identical mechanism. The important question here is whether the ratio of effectiveness of the compounds is the same on organisms rich in carboxylase (e.g., staphylococci) as it is on organisms containing no carboxylase (e.g., pneumococcus).

Wood and Austrian (282) reported that the action of carboxylase *in vitro* is

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[29] The enzyme carboxylase contains the thiazole ring and catalyzes the decarboxylation of pyruvic acid.

unaffected by a 0.0004 M (10 mg %) solution of ST, a concentration 50 to 200 times the concentration of coenzyme. This failure was no doubt due to the low concentrations of sulfonamide used for the amount of coenzyme present. Sevag and Shelburne (246), using 100 mg of air-dried brewer's yeast which was washed with alkaline phosphate to eliminate cocarboxylase while the specific protein of carboxylase remained as a source of specific protein carrier for carboxylase activity, found that approximately 2700 times more ST (by weight) than cocarboxylase was required for about 65 per cent inhibition of the latter's activity. The inhibition was observed to decrease as the amounts of cocarboxylase were increased. This is interpreted as showing a competition between ST and cocarboxylase for carboxylase protein. In such a system, thiamin exerted no antagonistic action, either by itself or in conjunction with coenzyme. Pyridoxin (vitamin B_6) failed to show any effect on ST inhibition of anaerobic respiration of E. coli, S. aureus, or brewer's yeast; thiamin had no counteracting effect on ST inhibition of S. aureus. ST inhibition of anaerobic respiration of S. aureus was antagonized by cocarboxylase at pH 6.2, which is the optimal pH for carboxylase activity, whereas at pH 7.16 no antagonism was obtained either in this organism or in E. coli. Evidently pH is an important factor here.

The inhibiting effect on yeast carboxylase was non-differentiable among SA, SP, SD, 2-aminopyrimidine, ST, sulfamethylthiazole, 2-sulfanilamido-5-ethyl-4-thiazolone, 2-aminothiazole; the exception was sulfamethyldiazine which was completely ineffective on the carboxylases of the organisms studied (251). The authors stated that though this supports the hypothesis that sulfonamide affinity may in part be related to structural similarity between components of the drug and the corresponding respiratory coenzymes, carboxylase could not be found in pneumococci, an organism whose growth is strongly inhibited by ST, so that ST must inhibit here by some other mechanism. At their face value these observations do not conclusively show that ST is a specific inhibitor of carboxylase in every instance. As already seen, there have been rather frequent indications that sulfonamides inhibit more than one enzyme.

The nature of the inhibition of the carboxylase system by ST appears to be an adsorption of the compound on the enzyme system in some way rather than on the substrate (246). Experiments were carried out as follows: in one case, ST (0.00414 M; 103 mg %) was allowed to be in contact with E. coli before pyruvic acid (0.05 M) was added, and after addition of the latter there was 34 per cent inhibition of anaerobic CO_2 production. In the second case, conditions were reversed: ST was in solution with the pyruvic acid, and the inhibition of the bacterial respiration after their addition was only 3 per cent. This indicates that pyruvic acid (the substrate) has a greater affinity for E. coli than ST when exposed to the bacteria at the same time. Of course, with the passing of time, the inhibitions observed in the two instances should approach one another as equilibrium is established.

A most important observation made by Sevag et al. (245, 246) is that PABA counteracts the inhibitory action of ST on the carboxylase activity of E. coli and S. aureus. PABA in sufficiently high concentrations is itself capable of inhibiting

the carboxylase activity of E. coli (246).[30]   The combined actions of ST and
PABA, in concentrations which are inhibitory separately, are not additive. This
may be interpreted in two ways.   First, the PABA may antagonize the ST action,
while the inhibition produced by the former persists[31]; this may well be, since the
sulfonamide-counteracting action of PABA and its inhibitory action in high con-
centrations might be dissociated phenomena.   Second, the separate inhibitions
may be of such a nature that they are not synergistic.   It is to be remembered,
however, that if such is the case the actions of both substances must be on the
same enzyme, since the carboxylase system is composed of only one rather than a
chain of enzymes (the various possible ways in which a substance could interfere
with such an enzyme will be discussed in F 2).   It is of interest to note that the
specific inhibition of the carboxylase system by acetaldehyde is not antagonized
by PABA (244).

Two things in particular evolve from the various results just described which
seem to be of extreme significance with regard to the mode of antagonism of sul-
fonamide action by PABA.   In the first place, the antagonism of the sulfonamide
inhibition of the carboxylase enzyme system by PABA scarcely can be explained
on the basis of the latter's functioning as an essential metabolite or substrate.
Secondly, in the experiments of Sevag et al. with live bacteria, the experimental
conditions were such that growth could not take place, yet antagonism of respira-
tory inhibition was obtainable.   Since the antagonism of the inhibition of
respiration of sulfonamides in the presence of PABA takes place in the absence
of growth, it is reasonable to conclude that the counteraction of the growth-
inhibiting effect of sulfonamides with PABA occurs through the pathway of
respiratory enzymes.

7. Certain Proposed Criticisms and Objections

Kempner (116) stated, "the fact that the inhibitory action of the sulfonamides
on pneumococcus and staphylococcus growth is unaltered, whether the bacteria
gain their energy by oxidation or by anaerobic frementation shows, just as does
the anaerobic p-aminobenzoic acid effect on sulfonamide-pneumococcus cultures,
that the sulfonamides do not act by way of inhibiting bacterial oxidation or fer-
mentation."   This interpretation does not seem to be warranted.   Fermenta-
tions or anaerobic respiratory processes are oxidative even though oxygen per se
is not involved, and it is well known that many substances are capable of inhibit-
ing anaerobic oxidative processes (50, 214).   As a matter of fact, it has been
shown that in yeast, which, like many bacteria, is capable of both aerobic and
anaerobic growth, growth is inhibited by narcotics to the same extent whether
under aerobic or anaerobic conditions (69).   Such a finding merely intimates that
the inhibitor is acting at a point in a respiratory chain which is common to both
aerobic and anaerobic oxidation.

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[30] Inhibition of bacterial growth by high concentrations of PABA has been referred to
in the previous section.   Sevag finds that these concentrations also inhibit bacterial
respiration, and that serum counteracts both the respiratory and growth inhibitions.
[31] This interpretation has also been suggested in certain instances of PABA antagonism
of sulfonamide inhibition of bacterial growth (288).

The inverse relationship existing between inoculum size and the sulfonamide concentration required for growth-inhibition has also been found to apply to respiratory inhibition (247). It has been the custom of several workers, including Ely (51) and Sevag and Shelburne (247, 248), to use very large inocula of bacteria (over one billion organisms per ml) in respiration experiments in order to obtain more reliable respiratory values. In order to produce inhibition of growth and respiration in such large inocula, sulfonamide concentrations 50 to 100 times the average therapeutic blood level were required. In the light of the observed inverse relationship, and the fact that the inhibition obtained under these conditions is completely reversible (both by PABA and by removal of adsorbed sulfonamide), there seems to be no reason why such inhibition should be different from that obtained with lower sulfonamide concentrations on fewer organisms. Wyss et al. (289) criticized this practice of using large sulfonamide concentrations, and claimed that their experiments with such concentrations indicated that results so obtained should not be regarded as an expression of typical sulfonamide activity. What they claimed for their experiments is probably true, for they used very small inocula (less than one-thousandth of the bacterial concentrations used by Ely, and Sevag and Shelburne) with the high sulfonamide concentrations.

Some workers have failed to obtain a respiratory inhibition of growing cultures of *Brucella melitensis*, hemolytic staphylococcus, and *E. coli* (86, 117, 118). The reason for these failures is not apparent. It should be remembered, however, that bacteria are notoriously susceptible to many factors and vary accordingly, and that experimental procedure in such experiments is as yet far from standardized. The answer must lie in future work and is of the utmost importance. Of the three criticisms of the "Inhibition of Respiration" theory which have been offered, this is the only one which cannot be satisfactorily rebutted. However, in view of the numerous and consistent reports of sulfonamide inhibition of bacterial respiration and the other considerations presented in this section, these few instances of failure to observe respiratory inhibition are not believed to constitute a serious obstacle. It must be remembered that oxygen is only one of the possible hydrogen acceptors in certain cellular oxidative reactions. There are many respiratory reactions in which acceptors other than oxygen participate. Therefore, the mere fact that no inhibition of oxygen consumption is observed under certain conditions is not in itself a disproof of an effect upon respiration.

8. Sulfonamide Inhibition of Oxidative Metabolism in Cells other than Bacteria

Plasmodia. SA markedly inhibits the oxygen consumption of *Plasmodium knowlesi* (a plasmodium producing a malarial infection in monkeys which is susceptible to sulfonamide therapy), but has no apparent effect on the anaerobic CO_2 production (34). The oxygen consumption of *P. cathemerium* is also inhibited by SA and ST (273).

Liver and Muscle. Chu and Hastings (31) found that SA concentrations corresponding to ordinary therapeutic levels (ca. 0.00075 M, 13 mg %) have no effect on, or slightly increase the oxygen consumption of rat liver and diaphragm. Higher concentrations (0.0075 M. 130 mg % and 0.038 M, 650 mg %) gave definite respiratory inhibition. This inhibition is antagonized by methylene blue

(194), indicating that the dye can function as a carrier at a point in a respiratory chain which is inhibited by the sulfonamide. Laves (147) also reported sulfonamide inhibition of aerobic respiration of diaphragm muscle, heart muscle brei, and liver brei. The inhibition of muscle respiration was antagonized by addition of coenzyme. That dehydrogenase activity is interfered with was demonstrated by the Thunberg technique. Laves stressed that, since only about 40 per cent of diaphragm muscle respiration goes *via* the cytochrome system, it is significant that sulfonamide can produce a 50 to more than 80 per cent inhibition of its aerobic respiration.

Bioluminescence. Bioluminescence in luminous bacteria and in the small Crustacean *Cypridina* is a result of the oxidation-reduction reaction of the luciferin-luciferase system, in which luciferin is the substrate and luciferase the enzyme. Johnson and Moore (111) found that SA in concentrations of approximately 0.006 M (100 mg %) readily inhibits bacterial luminescence (*Achromobacterium fischeri*, *Photobacterium phosphoreum*, *Vibrio phosphorescens*, and others were used in the following studies) in a manner resembling that of narcotics in general. The inhibition of luminescence appeared at a slightly lower concentration than growth-inhibition. *PABA* over a wide range of concentrations had no appreciable antagonistic effect on SA inhibition, and in fact, in high concentrations, added to it. These results were obtained with mature or washed cell suspensions. Later, however, PABA was found to antagonize SA inhibition of growth and light production in growing cultures of luminous bacteria (105, 107). The success of PABA in antagonizing sulfonamide action on actively growing cultures as compared to its failure with resting cells may be of some significance.

Johnson *et al.* (106, 110) showed that SA falls in a group of narcotics with the barbiturates, chloral hydrate, and PABA, which decrease the light intensity of luminous bacteria apparently by a chemical or adsorptive combination with an enzyme, since the inhibition is irreversible by pressure, although reversible by removing the inhibitor. Approximately one molecule of SA combines with a molecule of enzyme.

In an intensive study of the relationships between the SA inhibition of bacterial luminescence and temperature, it was noted that the heat of reaction for the combination of PABA with the enzyme luciferase appears to be approximately 4,000 calories higher than that for SA. This led to the expectation that PABA would combine about 1,000 times more readily than SA with the enzyme; actually, however, more PABA was required to produce an inhibition than SA. Thus, either the calculated heats of reaction are not real or PABA loses much more entropy in the process of adsorption than SA, which would be explained if the ionized form only of PABA and the undissociated molecules of SA are adsorbed (a situation apparently not true with sulfonamide inhibition of bacterial growth, at least). Concerning PABA antagonism of SA inhibition of bacterial growth, it was suggested that, if PABA, in its combination with the bacterial growth enzyme as normal substrate, has a heat of reaction greater than that of the SA combination, and the entropy change is approximately the same, it is quite understandable why only a small concentration of PABA is required for the an-

agonism; for approximately each 1,300 calories difference, PABA should combine en times more readily than SA. In luminescence, however, even if PABA combined with the luciferase preferentially in the presence of SA, it was reasoned that he SA inhibition would not be antagonized, since PABA cannot take the place of he normal substrate, luciferin, in the reaction. The result that would be expected, and which in fact was observed, of the combined action of PABA and SA would be an increased inhibition. This line of reasoning is based on the assumption that PABA is the normal metabolite of the bacterial growth enzyme inhibited.

SA (0.003 M; 50 mg %), ST (0.0006 M; 15 mg %), SP (0.001 M; 25 mg %), PABA (0.005–0.05 M; 70–700 mg %), and urethane (0.52 M; 2200 mg %) each independently and reversibly decrease the velocity constant of luminescence of the purified luciferin-luciferase system of *Cypridina* without decreasing the total light (108). The results showed quite satisfactorily that, in this system at least, the action of inhibitor is on the enzyme (luciferase), not on the substrate (luciferin), and in addition that a competitive action between substrate and inhibitor is not involved. The separate inhibitions by SA and PABA were partially additive when the two were present simultaneously. No PABA sulfonamide-antagonistic effect was seen at the concentrations used.

Sea Urchin (Arbacia) Eggs: That SA inhibits oxygen consumption and cell division of sea urchin eggs in a manner practically indistinguishable from typical narcotics was shown by Fisher and Henry (67) and Fisher *et al.* (68). Because of the important bearing that this work has on the "Inhibition of Respiration" theory of the mode of action of sulfonamides, and because of the development of these ideas in section F, it seems apropos that this work be presented in some detail:

Eggs of *Arbacia punctulata*, an echinoderm, are ideal to work with from many standpoints, not the least of which are their self-sufficiency in nutrition and the fact that cell division can be initiated at will by fertilization. Analysis of the action of narcotics (urethane and chloral hydrate) on respiration and cell division in the fertilized eggs and on respiration of the unfertilized eggs led to the interpretation that in the unfertilized *resting* egg one respiratory system is functioning, and that upon initiation of division by fertilization a second respiratory system is added in parallel to supply the energy for cell division (the respiration of the sea urchin egg increases upon fertilization). It was proposed that when a substance interferes with the latter system cell division is inhibited, and that narcotics inhibit this system considerably before the other,—thus accounting for the relatively little effect of narcotics on respiration as compared to cell division. The other respiratory chain, the one which is present in the inactive unfertilized egg, supplies the energy utilized in maintaining the cells in a basal state. SA, in the range of concentrations from 0.0005 M (9 mg %) to 0.04 M (690 mg %), was found to inhibit the respiration and division of fertilized eggs, but had no effect on unfertilized egg respiration (except in the very highest concentration possible, 0.04 M). This suggested that SA was, in the concentrations used, inhibiting practically specifically the respiratory system or chain (termed the *activity system*) fur-

nishing the energy for cell division. Further confirmation of this was obtained by employing combinations of inhibitors. Azide had no effect on respiration in the unfertilized egg and produced a maximum inhibition of fertilized egg respiration of about 50 per cent. On the possibility that azide might be inhibiting the activity system specifically, azide-SA and azide-narcotic combinations were applied to fertilized eggs. In both cases it was found that, over the range of concentrations of SA and narcotic which presumably inhibit the activity system when used individually, there was no additive inhibition when azide was used in combination at a concentration sufficient to produce the maximal effect of azide alone. From these two separate lines of evidence, it was concluded that SA action in this instance is indistinguishable from the action of typical narcotics. Thus, it becomes evident that it is not inhibition of the overall total respiration of a cell which is significant in inhibition of cell division, but rather the inhibition of that fraction which is specifically concerned with providing the energy for cell division.

PABA over a wide range of concentrations exhibited no sulfonamide-counteracting effect; in fact, in higher concentrations, it inhibited cell division and had a tendency toward additive inhibition with SA (68).

Since, in its gross details, the respiratory metabolism of bacteria is very similar to that of most other types of cells, the results cited above must be assumed to provide evidence in favor of the hypothesis that growth-inhibition is a result of inhibition of respiratory processes.

9. Neutralization by Respiratory Enzyme Factors of the Growth-Inhibition Caused by Structurally Related Inhibitors

The antagonizing effects of the group of substances primarily related to the respiratory systems on sulfonamide growth-inhibition will now be considered. It is perhaps unfortunate, that in all the experiments to be discussed below respiratory measurements were not made in parallel with the observations on growth. Due to this fact, the following experiments on bacterial growth as a basis for correlation with the theory of respiratory enzyme inhibition should be considered with some degree of reservation.

Staphylococcus aureus requires nicotinic acid or nicotinamide for the synthesis of coenzyme; preformed coenzyme also satisfies this requirement for growth.[22] There have been reports that coenzyme I but not nicotinic acid is able to antagonize sulfonamide inhibition, and this has been interpreted to mean that the sulfonamide is preventing the synthesis of the coenzyme from nicotinic acid (250, 282). In these experiments nicotinamide was not tested, so that it is impossible to tell whether there was inhibition of coenzyme synthesis from nicotinamide or whether there was inhibition of nicotinic acid conversion to its amide (46). The

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[22] Experiments on the effects of nicotinic acid on sulfonamide action may be much more complicated than is at first apparent. Kligler *et al.* (123) have recently found that, when nicotinic acid is lacking in a medium otherwise suitable for growth, the addition of glucose inhibits the growth of organisms (e.g., *Proteus*, dysentery bacilli, staphylococci) which are able to ferment this sugar in the presence of nicotinic acid.

latter is a definite possibility, since there is evidence that pyridine-3-sulfonic acid inhibits this conversion (170). Furthermore, the results obtained by Dorfman and Koser (indicating that the respiration of dysentery bacilli stimulated by DPN is inhibited in the same manner as the respiration stimulated by nicotinamide) do not support the idea that only DPN-synthesis is interfered with. This can also be interpreted as indicating that sulfonamides are blocking the dehydrogenase activity of these bacteria.[33] Some investigators have failed to obtain antagonism by coenzyme with this organism (263). The explanation for these conflictions in results is not apparent, but it might be pointed out that coenzyme preparations obtained from natural sources may be contaminated with other substances.

There is other evidence that sulfonamide does not inhibit coenzyme synthesis. Axelrod (269) reports inability to find any effect of SP on the synthesis of coenzyme I from nicotinic acid by red blood cells in vitro. Nicotinic acid, nicotinamide, and coenzyme I have been reported by Teply et al. (269) as antagonistic for SP inhibition of the growth of Lactobacillus arabinosus. Teply et al. concluded that their data presented no evidence that SP inhibits coenzyme I synthesis, and that it seems more probable that the function of the coenzyme is interfered with. In these experiments, however, apparently no allowance was made for the stimulation of growth by the antagonists in the absence of SP.

The crux of the question whether or not the sulfonamides with side groups specifically inhibit coenzymes containing an analogous group, as already outlined, depends on whether or not the inhibition by the structurally unrelated compounds can be antagonized by the addition of the particular coenzyme or enzyme component to the same extent as those compounds which are related. It has been shown by Wood and Austrian (282), using S. aureus, that nicotinamide and coenzyme I antagonize equally the action of unrelated compounds and the related SP. The antibacterial actions of methylene blue and thionine are also antagonized by coenzyme I.[34] Moreover, the antagonizing effects of nicotinamide and coenzyme are directly proportional to their ability to stimulate growth in synthetic media. In the case of E. coli, where these compounds do not enhance growth, they fail to counteract sulfonamide action (95, 128, 262, 282). Thiamin, riboflavin, pyridoxin, pantothenic acid, adenylic acid, ascorbic acid, inositol, choline and biotin exhibit no antagonism toward sulfonamide action on the vari-

[33] Mann and Quastel (185) demonstrated the presence of a hydrolytic enzyme, coenzyme I-nucleotidase, in fresh brain tissue responsible for the breakdown of the coenzyme, and which can be completely inhibited by high concentrations of nicotinamide. The stimulating effect of nicotinamide on lactic dehydrogenase activity of tissues in the presence of the nucleotidase is not a result of synthesis of nicotinamide to the coenzyme, but due to a competition of nicotinamide with the coenzyme for the nucleotidase. If a similar situation exists in bacteria, the interpretation of experiments using nicotinic acid, nicotinamide and coenzyme as sulfonamide-antagonistic factors is rendered even more difficult.

[34] It is known that these dyes serve as acceptors for dehydrogenase systems. These inhibitory effects must arise from the higher concentrations used. The counteraction of their inhibitory action by coenzyme I may indicate that it has greater affinity for its specific protein than these dyes possess. These facts also indicate that dehydrogenase activity of these bacteria must be restored before any growth processes can take place.

ous organisms which have been studied (95, 128, 259, 263). The above facts would seem to indicate that acceleration of growth by these vitamins, etc., are not determinant factors in antagonizing the sulfonamide action. Of distinct interest, however is the ability of thiamin to antagonize sulfonamide inhibition of fungus growth (147) and the development of paresis in pigeons on a sulfonamide-containing diet (52). Sulfonamides are reported as capable of producing (presumably directly) a peripheral neuritis (13), and it is well known that thiamin deficiency can also cause this.

McIlwain (170) has emphasized that in so complex a process as the utilization of nicotinic acid, part of which at least is for synthesis of pyridine coenzymes, many reactions must exist. Accordingly, unless the action of inhibitors is confined to one reaction, or several reactions are inhibited in the same manner, it is to be expected that several types of inhibition will be observed. He studied the inhibitors, pyridine sulfonic acid and its amide, and the antagonistic effects of nicotinic acid, its amide, and coenzyme I on *Staphylococcus sp.*, *Proteus sp.* and *E. coli*. Pyridine sulfonic acid inhibited the growth of *Proteus sp.* when nicotinic acid was the growth-promoter, but not when nicotinamide was used. As pointed out, this would suggest that the conversion of nicotinic acid to its amide is blocked. But growth promoted by coenzyme I was very strongly inhibited by pyridine sulfonic acid. It was considered as evident, therefore, that the fate of nicotinamide is not solely coenzyme I or its derivatives. Furthermore, it would appear that part of the block at least is at the degradation of the coenzyme to simpler units used in some synthesis (as already stated, nicotinamide itself may prevent such a degradation). Staphylococcus growth presented a similar picture: pyridine sulfonamide inhibited nicotinamide-promoted growth even to a greater extent than that promoted by the acid. These inhibitions were competitive, whereas the inhibition of coenzyme-promoted growth was not strictly competitive, and might represent superposition of competitive and non-competitive types of inhibition. The inhibition produced by the sulfonic acid was always of this latter type. Though this is a perfect example of how complicated inhibitor action may be, it is relatively clear that these inhibitors are specifically affecting the utilization of nicotinic acid or its derivatives.

There have been several reports on experiments *in vivo* which are very interesting and would seem to have an important bearing on this general question. Adenine sulfate[35] has been found to counteract the therapeutic action of SA, SD, SP, and ST on hemolytic streptococcal infection in mice (190). It was suggested (without experimental data) that these sulfonamides interfere with the normal utilization of adenine in these cases. Raiziss *et al.* (218) were able to obtain only a slight antagonism of SA and SP action on pneumococcal and hemolytic streptococcal infection in mice by nicotinic acid and vitamin C, the latter being given

[35] Adenine is a component of coenzymes I and II, and flavin adenine dinucleotide, in other words a component of dehydrogenases and flavoprotein enzymes. It is also a component of nucleoproteins of the living cells. These facts no doubt account for its being not only a possible essential nutrient for certain bacteria, but also offer a basis for specific affinity for bacteria, and therefore potential competition with inhibitors which exhibit affinities for the above adenine-containing enzymes of bacteria.

in the same amount as that of PABA which counteracted the sulfonamide action completely. This does not, therefore, preclude complete antagonism of sulfonamide action by nicotinic acid, or by vitamin C for that matter, at titrated dosage levels.

At present there is insufficient indisputable evidence to conclude more than that, in some bacteria, sulfonamides interfere with nicotinic acid metabolism. But again we are met with the paradox that in no single case of sulfonamide growth-inhibition has PABA been reported to fail in completely counteracting the process.

10. Resemblance of Sulfonamide Action to That of Indifferent Inhibitors

The sulfonamides, in their action, resemble indifferent inhibitors as exemplified by narcotics—the term narcotic being used in the sense that a cell function (growth and division in the case of bacteria) is being inhibited. If sulfonamides exercise their inhibitory effect by adsorbing onto specific proteins of respiratory enzymes (dehydrogenases) then their action is that most widely ascribed today to typical narcotics (50).[36] Narcotics have been observed to exert an inhibition of bacterial respiration (69, 275).[37]

The action of narcotics on cells is identical with that of sulfonamides; they both

[36] The present status of the mechanism of narcosis has been recently summarized by Fisher (65).

[37] This hypothesis of narcotic-like action raises a very interesting question: if sulfonamides bring about their therapeutic effect *in vivo* by narcotizing bacteria, why is not the animal host narcotized? Apparently the bacteria are susceptible to sulfonamide at a much lower concentration than the tissues of the host—a relation not existing with our ordinary narcotics and anesthetics. It is a well known observation that in order to produce a specific drug effect on different cells, widely different concentrations of the drug are often required. Zeller (291) found, for example, that the affinity of SA for the enzyme cholinesterase varies considerably with the source of the enzyme, even from organ to organ.

As a matter of fact there is some evidence suggesting that the host's tissues are in a state of basal anesthesia while on sulfonamide therapy, e.g., the effects of narcotics and anesthetics are potentiated by sulfonamide therapy (26). That sulfonamide therapy produces a slight sedative action in which alertness and judgment are impaired is a well-known clinical fact (160). For example, aviators are being grounded while receiving such treatment. Moderate doses of SA activate first and later depress the central nervous system of mice in the manner of a partial narcosis (267). In acute poisoning by the sulfonamides depression of the central nervous system is probably the most spectacular and consistent of the effects seen (156). That sulfonamides show a certain predilection for cells of the central (and peripheral) nervous system has also been indicated in studies showing degenerative cellular changes as a result of toxic doses of the drugs (13). Further evidence of the central nervous system depressant action of sulfonamides is the observation that SA, SP, and SD are capable of depressing the electrical activity of the monkey brain as measured by the electroencephalogram; ST, on the other hand, produces excitation accompanied or followed by convulsions (104).

It must be admitted, however, that some or all of such toxic reactions of sulfonamides *in vivo* may be produced by a mechanism totally unrelated to that of bacterial growth-inhibition. Thus, phenylsulfonamide, which has no *p*-amino group, is several times as toxic to mice as SA and brings about a similar picture of intoxication; the toxicity of acetyl sulfonamide is also similar (224).

are general cell inhibitors. As already seen, there have been several investigations (bioluminescence and sea urchin egg studies) in which the resemblance between sulfonamides and narcotics has been noted.

Summary: Sulfonamides inhibit the aerobic and anaerobic respiration of bacteria and other cells, whether in a resting state or actively dividing. When sulfonamides inhibit actively dividing cells, the inhibition of growth is directly related to the inhibition of respiration, indicating that the respiratory inhibition is responsible for the growth-inhibition. The identities of the inhibited respiratory enzyme or enzymes responsible for the growth-inhibition are not definitely known, but it has been shown that sulfonamides inhibit certain dehydrogenases and carboxylase, and it seems fairly certain that it is inhibition of this coenzyme-protein type of enzyme which secondarily results in growth-inhibition.

F. THE MECHANISM OF THE SULFONAMIDE INHIBITION OF CELL DIVISION

At present, there seem to be only two incontestable fundamental facts regarding the action of sulfonamides on bacteria, namely, sulfonamides inhibit bacterial multiplication, and certain substances antagonize this inhibition. We consider now some of the mechanisms which could possibly account for these two phenomena. The most widely considered hypothesis to date for the inhibition (that of Woods and Fildes) involves the antagonism of inhibition by PABA and in fact has been made to depend on the antagonism by PABA. It is therefore appropriate in considering the mechanism to commence by considering pertinent facts with regard to this antagonism.

1. Possible Ways in which PABA and Other Antagonists Could Counteract Sulfonamide Action

In recapitulating the following possibilities of antagonist action it is not pretended that the list is exhaustive. This is practically a virgin field in enzymology, and it is quite probable that mechanisms of inhibitor-antagonism are existent which are as yet unknown or unthought of.

a. Antagonists might act catalytically in promoting removal of sulfonamide from the cell. This was considered as a possibility for PABA antagonism by Woods (284). With what is known today regarding PABA antagonism this possibility seems remote. There is no evidence that any sulfonamide-antagonist works in this manner.

b. Preferential oxidation of sulfonamide-antagonists. This assumes that sulfonamides in order to be active must first be oxidized and that the antagonists are more easily oxidized than the sulfonamides thus preventing their activation. The primary assumption here has been definitely proved to be fallacious (section C); sulfonamides do not undergo an oxidation in order to become active.

c. Antagonist as a catalyst. This was proposed for PABA by Kohn and Harris (section D1g); according to this idea, sulfonamides compete with PABA in the reactions which the latter catalyzes. The antagonistic action of other substances on a basis of their being catalysts would be difficult to conceive; according to the scheme of Kohn and Harris these substances, such as methionine, occupy posi-

tions in reactions which are secondary to that catalyzed by PABA. The recent evidence, indicating the possible presence of PABA or a similar substance in bacteria, would seem to make this a possibility, but certain actions of PABA such as the antagonism of sulfonamide dehydrogenase inhibition cannot be so explained; this, along with other criticisms presented in section Dlg, restricts the acceptance of this viewpoint.

d. Direct interaction between sulfonamide and antagonist, forming an inactive complex. This cannot account for the counteracting action of PABA or methionine, but other substances, including urethane, ethyl alcohol, butyl alcohol, chloroform, ether, acetone, glucose, urea, peptone, albumin, bacterial protein, glycocoll, arginine, and hypoxanthine, have been shown by one method or another to combine more or less with sulfonamide (section D). These substances are of two categories, namely those which do and those which do not take part directly in metabolism. Apparently this mechanism can account for the antagonism by urethane, but whether or not the antagonism displayed by such substances as protein, peptone, and certain nutrient substrates is solely a result of inactive complex formation cannot be said. In such instances, it is quite possible that more than one mechanism is in effect.

e. Unspecific growth-stimulation. PABA, methionine, and peptone are among those antagonists whose activity cannot be explained on such a basis (section D). Amino acids and glucose undoubtedly antagonize sulfonamide inhibition by this mechanism. Under appropriate conditions, other individual substances acting as nutritive or oxidizable substrates will undoubtedly be shown to antagonize sulfonamide inhibition.

f. The Antagonist as an essential metabolite, competing with the sulfonamide for the enzyme. This was the role ascribed to PABA by Woods and Fildes (section D). The evidence for and against this proposal has already been presented, but in view of the importance of establishing whether this proposal is adequate, let us collect and examine this evidence in detail.

i. For: The relation between PABA and sulfonamide is a competitive one.

Against: Eyster's work (54) on charcoal adsorption and diastase activity results in strong doubt that the antagonism of sulfonamide by PABA is peculiarly significant, i.e., there is really no reason to think that PABA is a special molecule in the cell from the mere fact that a competition exists. Similarly, PABA antagonizes the sulfonamide inhibition of respiration (section E) and the inhibitions of the enzyme luciferase, an oxidative-reductive enzyme (section E8), and carboxylase (section E6). Thus, sulfonamide inhibits systems in which PABA is not used and the inhibition is still removed by PABA.

ii. For: Structural similarity between sulfonamide and PABA.

Against: Sulfonamide inhibition is antagonized by substances without structural similarity (section D). Peptone and amino acids containing aromatic groups (tryptophan, tyrosine, and phenylalanine) also inhibit SP adsorption on activated charcoal (146).

iii. For: PABA is a growth factor for some organisms.

Against: Only a growth factor in a few cells, which with one possible exception

(diphtheria bacilli, the pathogenicity of which was not reported; section D10) are non-pathogens, and these are less exacting in their growth requirements than the pathogens.   Microbiological assays and sulfonamide-antagonistic assays, the specificity of which may be questioned, have indicated that PABA is widely distributed in and is a product of living cells, including bacteria.   These assays should be repeated by exact chemical methods.   The growth-factor activity and sulfonamide-antagonistic activity of PABA cannot be assumed to be associated phenomena, since at least one instance is known where a substance even more active than PABA in growth-factor activity is completely devoid of sulfonamide-antagonistic activity (section D1d).   In instances where PABA is a growth-factor, it should be used up during sulfonamide-antagonistic action, thus allowing inhibition eventually to reappear.   This has never been reported.

iv. For: The probable presence of PABA in all cells; PABA antagonizes sulfonamide inhibition of almost all synthesizing cells which have been tried.

Against: Same objections as under *iii*.

v. For: The fact that other growth substances antagonize inhibitions by certain analogs.

Against: Same as under *ii*.   There is no doubt that some inhibitor analogs compete with substrates, but mere similarity in structure does not necessarily indicate such a relationship.

vi. For: Stimulation of PABA production caused by the presence of sulfonamide.

Against: The data are conflicting (section D1e).

vii. Against: Inhibition of other enzyme systems:[38] Respiratory systems (coincident with inhibition of growth, and also in the absence of growth, aerobic and anaerobic); respiration of bacteria, the sea urchin egg, muscle, liver, etc. (section E); carboxylase (section E6), carbonic anhydrase (section B3g); cytochrome oxidase (25), succinoxidase, luciferase (section E8), tyrosinase (11), cholinesterase (291), sucrase (53), amylase (53), diastase (54), protective proteinases (1).   The Woods-Fildes idea is untenable for the sea urchin egg since PABA in this case does not antagonize sulfonamide inhibition.

There is thus good reason to accept the idea that PABA normally enters as an intermediate or end product in the metabolism of many diverse types of cell, including bacteria (final judgment on this must be reserved until PABA is definitely identified in these cells by conclusive chemical methods).   However, there is clearly much evidence to show that this relation to normal metabolism is not necessary in order that PABA shall antagonize an inhibition by sulfonamide, i.e. PABA will antagonize sulfonamide inhibitions of enzymes whether or not PABA has any effect of its own on the enzyme system.   *As the question of PABA as an essential metabolite does not need to enter in these cases, it may be very seriously*

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[38] The following enzymes have been reported as not being inhibited by sulfonamides: catalase, peroxidase (section C); cytochrome oxidase, polyphenol oxidase, xanthine oxidase, uricase, urease (184); glucose dehydrogenase (Thunberg method), glyceric acid dehydrogenase (Thunberg method), *d*-amino acid dehydrogenase (section E5); pepsin, trypsin, serum di- and poly-peptidase (section D2a); phosphatase (53).

questioned whether such a consideration need be introduced in any case. This point is further stressed by the fact that antagonism occurs with many compounds other than PABA.

It would therefore appear, in the light of the additional data brought to light since the initial observation by Woods and Fildes, that there is very little basis for the presumption that sulfonamides inhibit growth by interfering with PABA metabolism specifically, or for that matter specifically with the metabolism of any analogous compound.

This consideration of course does not rule out the idea of sulfonamide specifically interfering with PABA metabolism. However, to be consistent one must apply similar consideration to methionine, peptone, protein, etc. In other words, the specific relation to PABA cannot be accepted unless at the same time a specific relation to these various other substances, which can be found in cells normally, is likewise accepted. The fact that the effect of PABA on sulfonamide inhibition is competitive cannot be used to support the idea that sulfonamide inhibits specifically a mechanism concerned with PABA metabolism, since, in the case of charcoal, diastase, etc., the antagonism between sulfonamide and PABA is competitive although sulfonamide is inhibiting a system which has no relation whatsoever to PABA. The fact, that methionine antagonism of the sulfonamide effect is not competitive (section D2a) while the PABA antagonism is competitive, does not of course indicate that the two antagonisms are not by the same mechanism. For it has long been recognized that inhibition of an enzyme may be competitive or non-competitive (89), and presumably therefore the effect of an antagonist on an inhibitor may also be competitive or non-competitive.

It is thus found that the Woods-Fildes theory, although consistent with certain observations, is not adequate for all observations.

g. Mutually exclusive action. As a corollary to the hypothesis that the action of sulfonamides is due primarily to an interference with the respiratory systems of cells (which will be considered in detail subsequently) and the observation that PABA antagonizes SA inhibition of respiration, it is necessary to imagine as Sevag and Shelburne (248) point out, that PABA in some way favors the removal of the SA from the catalytic system involved without itself interfering in the operation of that system.

Sevag *et al.* (246) quote the following two examples in which an enzyme is protected from one inhibitor by another inhibitor: Hopkins *et al.* (99) found malonate and succinate to prevent the inactivation of succinoxidase by oxidized glutathione. Protection by succinate could be relatively easily understood because it is the substrate for the enzyme, and it could be that the inhibitor in this case is competing with the substrate for the same activation center on the enzyme, but malonate itself is an inhibitor for the enzyme. Potter and Dubois (217) have recently shown in addition that succinate antagonizes various sulfhydryl inhibitors such as quinone and *p*-phenylenediamine, cysteine and cystine, and that malonate, while producing a definite inhibition itself, prevents any additive inhibition by quinone or *p*-phenylenediamine. They interpret the latter phenomenon as one of inhibitors whose actions are "mutually exclusive,"

although each of the two types of inhibitor combines with the enzyme at a different locus. Thus malonate (and also succinate) shields the enzyme from sulfhydryl reactants; apparently the presence of the former makes it impossible for the latter to approach its combining site on the enzyme.

Dixon and Keilin (45) demonstrated the complete protection afforded xanthine oxidase aerobically and anaerobically from cyanide by uric acid, adenine, guanine and hypoxanthine. Neither uric acid nor guanine had any effect on the enzyme in the absence of cyanide. The protection afforded this enzyme by hypoxanthine was at first thought to be an example of a substrate protecting its enzyme against an inhibitor by not allowing the latter's approach. This explanation proved to be inadequate, for neither hypoxanthine nor the hydrogen-acceptor alone could confer protection, complete protection being obtainable only when both were present, i.e., when the oxidation of hypoxanthine was actually proceeding. The other substances gave protection without the presence of the hydrogen-acceptor.

The "mutually exclusive" action as originally described above was proposed for an instance in which the substance providing the shielding action was producing an inhibition *per se* through its very shielding action. It seems quite plausible that such a shielding may occur in some instances in which an inhibition does not result from the shield, i.e., adsorption of the substance giving the protection does not inhibit the enzyme activity (at that concentration at least).

Some such mechansim must be the explanation for the observations made by Eyster (54) on the charcoal model and on the digestion of starch by diastase. In the charcoal model, PABA counteracted SA inhibition of the adsorption of methylene blue, this antagonism being definitely shown as competitive. When the PABA concentration was increased sufficiently an additive inhibition was seen. In the case of diastase activity, PABA antagonized SA inhibition and *vice versa*, thus emphasizing the fact that a balance exists between the two substances.

There are many examples in cell physiology where two substances, separately toxic, completely or in part nullify each other's effects when mixed in proper balance, e.g., copper and calcium ions. Of extreme interest and pertinence is the finding by Valko and Dubois (272) that relatively harmless surface-active cations can completely antagonize the antibacterial action of highly toxic cations. These authors explain the phenomenon by what they call "ionic exchange", i.e., the harmless cations compete successfully with the toxic cations for the same spaces (presumably the carboxylic groups of the protein material). Duponol was found to antagonize the antibacterial action of acriflavin and, as pointed out by Valko and Dubois, it is unnecessary to assume that sodium dodecyl sulfate can act as an essential metabolite, an explanation favored by McIlwain (172) for the antagonistic action of tryptic casein, nucleic acid, and yeast extract for acriflavin. These observations offer a close parallel to those of Eyster on the charcoal model. Again, however, we face the question: since the toxic cation is toxic presumably because of an adsorption onto some specific locus, why is not the nontoxic cation toxic, when and if it combines at the same locus? This cannot be answered as

yet with any great degree of satisfaction, but some such mechanism as already described may well be in operation.

Other examples of "mutual exclusion" apropos the problem with which we are chiefly concerned, namely, antagonism of sulfonamide inhibition, have been reported:

SA antagonizes the bacteriostatic effect of mercuric ion and *vice versa* (136).

PABA counteracts SA inhibition of light production in growing cultures of luminous bacteria (E8).

PABA antagonizes ST inhibition of carboxylase (245). With this particular enzyme system, concentrations of PABA which alone inhibit do not produce an additive inhibition when added to inhibitory concentrations of ST; thus here the ST and PABA inhibitions are "mutually exclusive" in the same way as those reported for the two types of inhibitors of succinoxidase.

PABA is capable of shielding bacteria from inhibitors other than sulfonamides, among them being germanin, neostibosan, arsphenamine, and neoarsphenamine (202).

It seems necessary to point out, however, that in certain instances the antagonism or shielding action of PABA may not be in any way related to its action as an inhibitor. Thus, it may be that its antagonistic action takes place in both low and high concentrations, but at the higher concentrations PABA acts also as an inhibitor by some unrelated mechanism.

The exact mechanism of these antagonisms is yet to be learned, but there is ample evidence that antagonism of toxic substances can occur under circumstances indicating conclusively that the antagonism is a physical, surface phenomenon. Such an interpretation must be made of PABA's antagonism in several instances, and thus far there has appeared no definite evidence why this should not apply in every instance.

2. Possible Ways in Which an Inhibitor can Stop Cell Division

At the present time there seems to be no disagreement that sulfonamides inhibit certain enzymes, and that this is undoubtedly the primary mechanism of their action on cells. Let us next examine the various mechanisms whereby an inhibitor can interfere with cell multiplication and see which one agrees best with what is known about sulfonamide action.

a. *Inhibition of one or more anabolic reactions which supply material (protoplasm) for increased cell mass.* This may or may not be a factor in cell division. For example, in the early divisions of fertilized ova there is frequently no increase in protoplasm, but rather a repeated subdivision of that already present to daughter cells which become increasingly smaller with each division. On the other hand, it has been observed that certain cell inhibitors inhibit division while the cell mass itself increases up to a certain point. Interference with anabolic reactions which form enzymes responsible for other anabolic reactions must also be considered.

Inhibition of an anabolic reaction supplying material for increased cell mass may be brought about in several ways:

i. Direct interference with the enzyme. This forms the basis of the Woods-Fildes theory of sulfonamide action. The considerations regarding this theory have already been presented. While there seems little doubt that sulfonamides interfere with enzymes, there is actually no definite evidence that the enzyme inhibited is specially concerned with PABA as substrate.

ii. Direct interference with the substrate. In contrast to SA and PABA which interfere with the enzyme luciferase, azide inhibits *Cypridina* luminescence by combining with the substrate, luciferin (28, 108). This is merely cited as an example of this type of inhibition. There is no indication that this mechanism could account for inhibition by sulfonamide.

iii. Interference with the infusion of raw materials or the effusion of waste products. Changes in membrane permeability brought about by sulfonamide action could interfere with the infusion of necessary substrates or the effusion of waste products. An increased concentration of such products may produce an inhibition by any of the other mechanisms outlined. Reaction products which are normally substrates for other reactions may accumulate to the point where they, because of the reversible nature of the enzyme reaction which formed them, cause the reaction to slow down and eventually come to equilibrium. Such an accumulation would be due to an inhibition of the enzyme system which utilizes these products as substrates. It must be remembered again, however, that it is an opinion held by many that the great majority at least of bacterial metabolic reactions take place at the cell surface.

iv. Prevention of formation of substrate. This obviously would be a secondary effect, the primary inhibition being on the reaction producing the substrate; this latter inhibition could be of any type.

v. Inhibition of the formation of the enzyme itself. Very little is known concerning enzyme formation, but it is conceivable that a sulfonamide could inhibit this formation in some way. Decreased enzyme concentration would result in decreased reaction rate unless an excess of the enzyme existed. The enzyme concentration would decrease with time, depending on the rate of its disintegration. This could explain the delay in sulfonamide action observed by many. There is some evidence that in certain instances sulfonamides hinder the synthesis of respiratory coenzyme (section E9).

b. Inhibition of one or more oxidative enzymes concerned with supplying energy for the production of increased cell mass or cell division or both. The possible validity of such a mechanism hinges upon whether or not the respiratory processes are inhibited during inhibitor action; and, as was seen in the case of sulfonamides, the observations on bacteria with relation to this mechanism have, with but a few exceptions, shown such an inhibition. The evidence in favor of the hypothesis has indicated that the enzyme systems affected are the dehydrogenases (protein-coenzyme systems). This is interesting, since it is these same enzymes which narcotics inhibit.

In interpreting results from respiratory experiments it must be remembered that a substance conceivably could interfere with dehydrogenase action in any one of several ways. On the basis of what is known about these enzymes (50)

one may list the possible modes of action of a dehydrogenase inhibitor as shown[39] in fig. 2.

$$\begin{array}{ccc} & \text{I} & \text{coenzyme} \\ & \nearrow\!\!\!\!\diagup & \Big\|\,\text{II} \\ \text{protein} & & \\ & \nwarrow & \Big\| \\ & \text{III} & \text{substrate} \end{array}$$
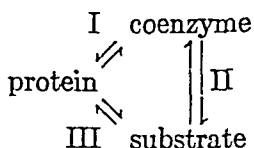
Fig. 2. Bonds connecting the three components of the enzyme–substrate complex denote reversible combinations. The enzyme is capable of functioning only when its two components, the coenzyme and protein, are associated.

    i. at locus I, by
        a. adsorption onto the protein,—competition with coenzyme for the protein.
        b. adsorption onto the coenzyme,—competition with the protein for the coenzyme.
    ii. at locus II, by
        a. adsorption onto the coenzyme,—competition with the substrate for the coenzyme.
        b. adsorption onto the substrate,—competition with the coenzyme for the substrate.
    iii. at locus III, by
        a. adsorption onto the substrate,—so that the protein is no longer specific for the substrate or unable to function with the substrate (would be competitive).
        b. adsorption onto the protein,—the protein is no longer functional.

It is conceivable that a substance may so change the oxidation-reduction potential of an oxidative enzyme system that it can no longer function in its normal capacity. This has been proposed for the action of azide on cytochrome oxidase (6). There are two possible ways in which this could be brought about. First, the substance added, if an oxidation-reduction system itself, could poise the enzyme system at a non-functional level depending on the relative amounts of the two systems present. Since, as concluded in section C, the sulfonamides do not become oxidized before or during their bacteriostatic action, this could not occur. Second, the substance could by combination with the enzyme in any of the ways outlined above cause a shift in the oxidation-reduction potential of the system. Thus we see that this latter proposal is fundamentally not an alternative mode of interfering with oxidative enzymes from those already presented.

The above approach at best is extremely hypothetical but serves its purpose if only to illustrate the complexity of the situation and the caution which must be exercised in interpreting certain types of experiments. The coenzymes are non-specific for a variety of substrates (it is the protein component which confers the specificity on the protein-coenzyme complex) (214). Further complications

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[39] Many enzymes other than respiratory enzymes are similarly composed of a protein component and a coenzyme; with such enzymes this consideration would also apply.

arise from the fact that, in the case of bacteria, enzyme (protein) saturation with coenzyme has frequently been found to be rather low and dependent to a great extent on the nature of the medium (78).

Laves (147), after demonstrating inhibition of aerobic and anaerobic respiration of liver brei and heart-muscle brei (section E8), supported the theory, which he apparently accredits to K. Mulli, that sulfonamides inhibit intracellular oxidation processes. Inhibition of enzyme activity was considered as probably resulting from either a direct poisonous action on the coenzyme, or what corresponds to mechanism i, a above. Sevag et al. (section E4) have also proposed what is essentially mechanism i, a for the inhibition of dehydrogenase activity by sulfonamides in bacteria. They have also suggested the possibility of the formation of an inactive "drug-protein-enzyme" complex which corresponds to iii, b. Similarly, Dorfman and Koser (46) suggested mechanism i, a for the SP and ST inhibition of the respiratory enzyme containing nicotinamide. That sulfonamides have a definite affinity for protein, which in magnitude is directly proportional to its bacteriostatic activity, is well-established (section D3a).

The mechanisms schematized so categorically above must not be taken too literally. Adsorption of a substance on the protein surface, say as in iii, b, may indirectly affect the relationship existing at locus (I) by change of electrical charge, polarity, steric hindrance, etc.

Sulfonamides stop cell growth and so do numerous other inhibitors, such as narcotics, cyanide, azide, etc. But of these, the action is more like that of the so-called "indifferent inhibitor" typified by the narcotics, dyes, etc. The effects of sulfonamides on bacteria and other cells are reversible for a time (section B3a) as are the effects of many indifferent inhibitors. Most typical of the indifferent inhibitors are the narcotics, and, as seen in section E10, there is much evidence to suggest similarities between the effects of narcotics and sulfonamides in forms higher than bacteria.

Concerning the similarity of sulfonamides to narcotics, it is typical of narcotics to inhibit function (e.g., cell division) completely with relatively little effect on respiration, i.e., inhibition of division increases with respiratory inhibition, but the former increases at a faster rate so that by the time division is completely blocked only a relatively small part of the overall normal respiration of the cell is inhibited. This of course does not mean that as the sulfonamide concentration is increased beyond that just required to stop division completely, respiratory inhibition will not also increase. As we have seen (section E), Sevag and others find inhibition of oxygen consumption by sulfonamides but, as with narcotics, it is relatively less than the inhibition of cell division (at or below the concentration of sulfonamide which just completely stops multiplication). Whence it is very likely that sulfonamides are blocking the energy production requisite for cell multiplication.

The complete picture of sulfonamide effect on oxygen consumption must account for the large oxygen consumption still left when cell division is completely stopped, i.e., the question arises as to what mechanism will account for

complete inhibition of cell division and very little inhibition of oxygen consumption or carbon dioxide production if these are significant at all in the action of sulfonamides. The suggestion proposed for the action of narcotics on various cells (65, 67, 69) would seem to apply here, and, in fact, is perhaps most clearly illustrated by the action of narcotics and SA on sea urchin eggs (see section E8). From this work it is evident that it is not inhibition of the overall total respiration of a cell which is significant in inhibition of cell division, but rather the inhibition of that fraction which is specifically concerned with providing the energy for cell division. Sulfonamides and narcotics inhibit this fraction more or less specifically, thus accounting for the complete inhibition of cell division and a relatively small inhibition of total respiration.

One must conclude, therefore, that an inhibition can be antagonized without the antagonist necessarily being a normal component of the reacting system in question. This fact destroys the force of the suggestion for the mechanism of sulfonamide action that, since PABA antagonizes sulfonamide action competitively, and since PABA is a growth factor for some cells, sulfonamides act by interfering with the metabolism of PABA.

Seeking now a more satisfactory explanation, and taking account of the newer investigations, there seems to be much reason for grouping the sulfonamides with such so-called indifferent inhibitors as the narcotics. In this connection, it has already been suggested that normal cell division depends upon the normal function of an unknown, but *specific fraction* of the total oxidative reactions of the cell, and that the indifferent inhibitors affect cell division by inhibiting that specific set of reactions. In keeping with this general consideration, Sevag and coworkers have claimed, and a recalculation of their data establishes, that oxygen consumption in bacteria is in fact inhibited by concentrations of sulfonamide which stop growth. In the sea urchin egg, the only case which has thus far been adequately investigated, and one which is more suitable for general investigation than are bacteria, it seems very clear that SA stops cell division by interfering with a specific fraction of the total oxidative metabolism of those cells. Accepting this view, it is probably necessary to couple with it the supposition that sulfonamide-antagonists exclude the sulfonamides from the cell catalyst or catalysts without themselves interfering with the action of those catalysts.

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THE TYPES MITIS, INTERMEDIUS AND GRAVIS OF CORYNEBAC-
TERIUM DIPHTHERIAE [Correction]

"In Professor J. W. McLeod's interesting review (1) on the three types of *Corynebacterium diphtheriae*, there is one point especially concerning our own work (2) which we should like to see corrected. In discussing atypical strains (page 9) which have a *gravis*-like colony but do not ferment starch (type IV of Wright and Christison), Professor McLeod wrongly attributes to us the finding that such strains in Australia had the same sensitivity towards bacteriophage as the predominant *gravis* strains, whereas, in fact, they did not, but had the same sensitivity as *intermedius* strains. In our experience these strains had the following characteristics: They did not ferment starch but resembled *gravis* in type of growth in broth; on the lysed-blood tellurite medium used by us, they displayed a distinctive colony type which approximated to the *gravis* type. Their serological reactions and phage sensitivity were, however, of the *intermedius* type."

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R. T. SIMMONS

G. ANDERSON

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"...with regard to a statement on page 9 of the review, ...it would have been better if my argument had been formulated somewhat in the form that: 'The findings of Keogh, Simmons and Anderson with bacteriophage which would place these strains in the *intermedius* type also brought out a possible relationship between them and the *gravis* type predominant in Australia but none to *mitis*'."

<div align="right">

J. W. MCLEOD

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(1) MCLEOD, J. W. 1943 The Types *mitis, intermedius* and *gravis* of *Corynebacterium diphtheriae*. Bact. Rev., 7, 1–41.
(2) KEOGH, E. V., SIMMONS, R. T., AND ANDERSON, G. 1938 Type-specific bacteriophages for *Corynebacterium diphtheriae*. J. Path. Bact., 46, 565–570.